The Role of Antibodies in Controlling Flavivirus Cell Entry

rijksuniversiteit groningen

The research described in this thesis was performed at the Department of Medical Microbiology, Molecular Virology section, University Medical Center Groningen, within the GUIDE Graduate School for Drug Exploration.

The printing of this thesis was financially supported by the University of Groningen, The Netherlands, the GUIDE Graduate School for Drug Exploration, Groningen, The Netherlands, INstruchemie B.V., Delfzijl, The Netherlands, and Clean Air Techniek B.V., Woerden, The Netherlands.

Cover illustration: Impression of a flavivirus particle within an endosome
Cover design: Bastiaan Moesker

ISBN
978-90-367-5045-5 (hardcopy)
978-90-367-5046-2 (digital)

Printed by Ipskamp Drukkers B.V. The Netherlands

RIJKSUNIVERSITEIT GRONINGEN

The Role of Antibodies in Controlling Flavivirus Cell Entry

Proefschrift

ter verkrijging van het doctoraat in de
Medische Wetenschappen
aan de Rijksuniversiteit Groningen
op gezag van de
Rector Magnificus, dr. E. Sterken,
in het openbaar te verdedigen op
woensdag 12 oktober 2011
om 12.45 uur

door

Bouko Jan Sander Moesker

geboren op 14 september 1984
te Groningen

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Learn now of things you must yourself admit
exist, and yet remain invisible

Lucretius, On the Nature of the Universe
Book one, Lines 269-270

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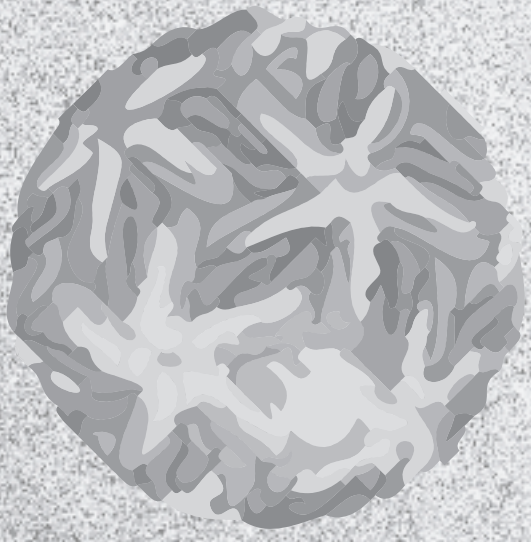
General Introduction



CHAPTER 1

GLOBAL BURDEN OF DENGUE VIRUS AND WEST NILE VIRUS

Dengue virus (DENV) and West Nile virus (WNV) are emerging mosquito-borne pathogens belonging to the *Flavivirus* genus in the family *Flaviviridae*. The past decades have witnessed a global resurgence of DENV, now causing an estimated 50 million infections annually (WHO, 2009). Some 2.5 billion people worldwide are at risk of being infected with DENV. Although most dengue cases are asymptomatic, infection may result in dengue fever, a debilitating febrile illness, or progress into more severe manifestations known as dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) (Gubler, 1998; Halstead, 2007). There are four dengue serotypes (DENV 1-4) all of which may cause similar clinical manifestations. Importantly, the development of severe disease is associated with secondary infections with a heterotypic DENV serotype (Halstead, 1970; Thein *et al.*, 1997; Guzman *et al.*, 2002). Co-circulation of multiple serotypes due to the global spread of DENV has been responsible for an increased number of epidemics (Gubler, 2002; Mackenzie *et al.*, 2004). WNV is common in regions throughout the Old World and Oceania where it causes incidental outbreaks associated with only minor illness (Mackenzie *et al.*, 2004). However, with the emergence of a more virulent strain of WNV lineage 1, outbreaks in the past decades have involved high numbers of neuroinvasive disease. In recent epidemics, approximately 1% of infections resulted in meningitis, encephalitis or flaccid paralysis, with fatality rates of ~10 % among hospitalized patients (Petersen *et al.*, 2003; Gubler, 2007). Many survivors experience long-term cognitive and neurological impairment (Petersen *et al.*, 2003; Mackenzie *et al.*, 2004). Moreover, WNV lineage 1 has recently emerged in North America and subsequently spread to South America and the Caribbean (Gubler, 2007). Currently, no vaccines or antiviral drugs are available to prevent or treat disease caused by DENV or WNV.

SCOPE OF THE THESIS

Antibodies (Abs) play a central role in controlling flavivirus infections. Individuals that have undergone flavivirus infection are protected upon reinfection due to the presence of protective Abs (Klockmann et al., 1991; Gubler, 1998). At the same time, the presence of Abs may predispose individuals to more severe disease upon infection with DENV (Halstead, 1970; Kliks et al., 1988; Thein et al., 1997; Guzman et al., 2002). A more fundamental insight into the molecular basis of protection against flavivirus infection is crucial for the development of antiviral strategies. Therefore, the studies described in this thesis focus on the role of the membrane fusion process in the cellular entry of flaviviruses, with the ultimate aim to gain a better understanding of the mechanisms of Ab-mediated neutralization and enhancement of flavivirus infection.

Chapter 2 reviews the steps involved in the receptor-mediated endocytosis of flavivirus particles with particular emphasis on the molecular mechanisms that govern the process of membrane fusion. Furthermore, the influence of particle maturation on the infectious properties of flaviviruses is discussed. Finally, the mechanisms by which Abs may neutralize or enhance and thus critically influence the outcome of flavivirus infections are addressed.

Chapter 3 presents a detailed characterization of the basic requirements for WNV membrane fusion with liposomal target membranes. This study provides a kinetic analysis of WNV fusion by monitoring the redistribution of fluorescent probes during membrane merger in an on-line fashion. It is shown that WNV fuses at mildly acidic pH and does not share the stringent lipid requirements of alphaviruses. Furthermore, it is demonstrated that fully immature particles can be rendered fusogenic upon *in vitro* maturation.

In **Chapter 4**, the inhibitory mechanism of the potently neutralizing anti-WNV monoclonal Ab (MAb) E16 is investigated. Using confocal microscopy, it is established that E16-opsonized WNV particles readily enter cells but are then targeted for lysosomal degradation. Subsequently, it is demonstrated that E16 strongly inhibits WNV fusion in a concentration-dependent manner. Moreover, fusion with liposomal target membranes could be completely blocked. These results suggest that MAb E16 inhibits infection by preventing fusion with the endosomal membrane.

In **Chapter 5**, the neutralizing properties of two potent anti-WNV MAbs raised during natural WNV infection are investigated. Both MAbs were mapped to epitopes that likely span the E dimer-interface. Binding affinity was shown to be pH-dependent, suggesting the involvement of a conformational epitope. In fusion measurements with liposomes, opsonization with these MAbs strongly inhibited the fusogenicity of WNV. This study shows that naturally occurring MAbs that bind epitopes outside of DIII of the E-protein can potently inhibit infection through blockade of membrane fusion.

Chapter 6 establishes a biological role for immature WNV in the presence of anti-prM MAbs. Otherwise non-infectious immature WNV particles were demonstrated to readily infect Fc-receptor bearing murine macrophages when opsonized with anti-prM MAbs. Infectivity was dependent on the activity of the endoprotease furin, as no infectious titer was observed upon addition of a furin-inhibitor to cells prior to infection with opsonized immature WNV. Moreover, WNV could be detected in the blood serum and brain of mice that received anti-prM opsonized immature WNV, while no virus was found in sera or brain from mice infected with immature WNV alone. The observation that antibody-opsonized immature WNV can cause mortality due to encephalitis in these mice suggests that immature virus should be regarded as an important component of flavivirus infection.

Chapter 7 provides evidence that MAbs directed against the E-protein can also enhance the infectious properties of immature flaviviruses. We found that opsonization with the anti-E MAb E53 rendered both immature DENV and WNV infectious in a furin-dependent manner. Interestingly however, while E53 readily promoted infectivity of immature DENV on murine P388D1 macrophages, the increase in infectivity was not observed on human leukemia K562 cells. It is shown that the ability of furin to cleave immature DENV is altered by the presence of MAb E53, possibly explaining the differences in enhancement observed in the two cell lines. The relevance of anti-E MAbs that preferentially recognize immature particles and their role in flavivirus disease pathogenesis is discussed.

Chapter 8 provides a summary and discussion of the findings described in this thesis.

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Flavivirus cell entry and membrane fusion

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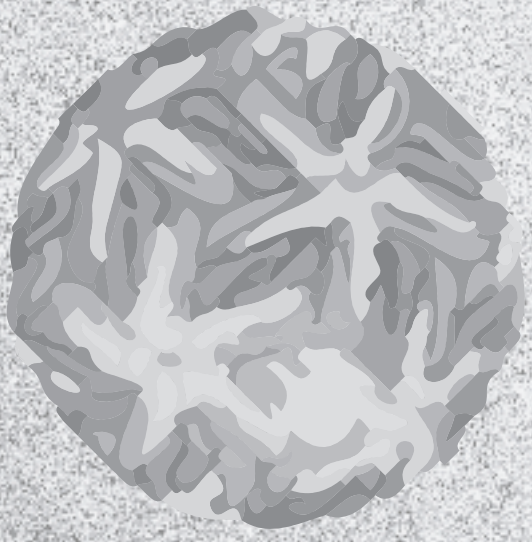
& Jan Wilschut

Modified from:
Viruses (2011), **3**: 160-171



Chapter 2

ABSTRACT

Flaviviruses, such as dengue virus and West Nile virus, are enveloped viruses that infect cells through receptor-mediated endocytosis and fusion from within acidic endosomes. The cell entry process of flaviviruses is mediated by the viral E glycoprotein. This short review will address recent advances in the understanding of flavivirus cell entry and will describe how antibodies control the infectious properties of the virus.

INTRODUCTION

Flaviviruses are enveloped arthropod-borne RNA viruses that belong to the family of the Flaviviridae (Lindenbach & Rice, 2003). The flavivirus genus comprises more than 70 viruses including, dengue virus (DENV), West Nile virus (WNV), tick-borne encephalitis virus (TBEV), Japanese encephalitis virus (JEV), and yellow fever virus (YFV). Flaviviruses can induce a broad range of clinical symptoms, ranging from mild illness to severe disease such as hemorrhage and encephalitis(Lindenbach & Rice, 2003). For most flaviviruses, lifelong protection is achieved after primary infection. In case of DENV, individuals are protected against re-infection with the same serotype. Intriguingly however, there are 4 distinct DENV serotypes, and multiple studies have shown that re-infection with another serotype sensitizes individuals for the development of dengue hemorrhagic fever (DHF) (Halstead, 1970; Burke *et al.*, 1988; Thein *et al.*, 1997; Guzman *et al.*, 2002; Recker *et al.*, 2009). Not only individuals experiencing a secondary infection with a heterologous DENV serotype but also infants born to dengue-immune mothers were found to be at greater risk for the development of DHF (Kliks *et al.*, 1988; Halstead *et al.*, 2002). This indicates that (maternal) antibodies play a crucial role in controlling the outcome of disease. Whereas homosubtypic antibodies are generally believed to protect the individual from disease, heterosubtypic antibodies have been suggested to mediate severe disease. The phenomenon that DENV opsonized with heterosubtypic antibodies can result in enhancement of viral infection is presumably related to the natural route of DENV entry into cells. Here we will give a brief overview on the route of flavivirus cell entry and how antibodies may neutralize or enhance viral infectivity.

STRUCTURE OF FLAVIVIRUSES

Flaviviruses are small, icosahedral viruses. The viral genome consists of a single-stranded, positive-sense RNA molecule which is complexed to multiple copies of the capsid protein (Lindenbach & Rice, 2003). The nucleocapsid is surrounded by a host-derived lipid membrane, in which two transmembrane proteins are inserted, the major envelope glycoprotein E (53 kDa) and the membrane protein M (8 kDa). The M protein is a small proteolytic fragment of its precursor form prM (approx. 21 kDa). In mature virions, the E glycoproteins are arranged in 90 homodimers with sets of three E head-to-tail homodimers that lie in 30 rafts and form a herringbone pattern (Kuhn *et al.*, 2002). The E ectodomain has three structurally distinct domains (DI, DII, DIII) that are connected by flexible hinge regions (Rey *et al.*, 1995). Under the E protein shell, 180 copies of the M protein are located which are anchored into the viral membrane by two transmembrane helices (Kuhn *et al.*, 2002; Zhang *et al.*, 2003a). Neither E nor M interacts with the nucleocapsid in mature virions (Mukhopadhyay *et al.*, 2005). In infected cells, virions are initially assembled in an immature form with a distinct structural organization. In these particles, the E glycoprotein is associated with the glycoprotein prM and three of these heterodimers form one viral spike (Kuhn *et al.*, 2002; Zhang *et al.*, 2003b). Virus particle maturation occurs during viral egress.

Chapter 2

FLAVIVIRUS CELL ENTRY

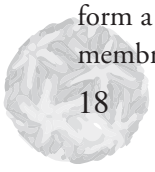
Receptor binding

The first step in the infectious cell entry pathway of flaviviruses involves binding of the E glycoprotein to a cellular receptor. Flaviviruses must recognize a ubiquitous cell surface molecule or utilize multiple receptors for cell entry as flavivirus infection has been observed in a variety of cell lines derived from different host species (Rodenhuis-Zybert *et al.*, 2010b). In recent years several attachment factors have been identified, indicating that flaviviruses may use multiple receptors for cell entry.

Negatively charged glycoaminoglycans, such as heparan sulfate, which are abundantly expressed on numerous cell types are utilized as low-affinity attachment factors by several flaviviruses (Chen *et al.*, 1997; Hilgard & Stockert, 2000; Mandl *et al.*, 2001; Germi *et al.*, 2002; Lee & Lobigs, 2008; Chen *et al.*, 2010; Kozlovskaya *et al.*, 2010). These interactions serve to concentrate the virus at the cell surface and are mediated by domain DIII of the E glycoprotein. Multiple other attachment factors have been identified for DENV in mammalian cells including heat-shock proteins 90 and 70 (Reyes-Del *et al.*, 2005), neolactotetraosylceramide (Aoki *et al.*, 2006), CD14 (Chen *et al.*, 1999), GRP78/BiP (Jindadamrongwech *et al.*, 2004), 37-kDa/67-kDa laminin (Thepparit & Smith, 2004), and C-type lectins such as DC-SIGN (dendritic cell-specific intracellular adhesion molecule-3 (ICAM3)-grabbing non-integrin) (Navarro-Sanchez *et al.*, 2003; Tassaneetrithep *et al.*, 2003; Lozach *et al.*, 2005), the mannose receptor (Miller *et al.*, 2008), and C-type lectin domain family 5, member A (CLEC5, MDL-1) (Chen *et al.*, 2008). In mosquito cells, DENV has been shown to interact with heat-shock protein 70, R80, R67 and a 45-kDa protein (Yazi *et al.*, 2002; Reyes-Del *et al.*, 2005; Mercado-Curiel *et al.*, 2006). Crystallographic studies on DENV-DC-SIGN complexes revealed that interaction with DC-SIGN is preferentially mediated through the carbohydrate moiety at Asn67 in EDII (Pokidysheva *et al.*, 2006). WNV has also been shown to interact with DC-SIGN and DC-SIGNR in dendritic cells (Davis *et al.*, 2006a; Davis *et al.*, 2006b). Furthermore, WNV, JEV, and DENV albeit to a lesser extent, have been documented to bind to αvβ3 integrins expressed on mammalian cells, mediated through interaction with EDIII (Chu & Ng, 2004b; Lee *et al.*, 2006). On the other hand, entry of WNV into embryonic mouse fibroblasts and hamster melanoma is independent on αvβ3 integrin binding, suggesting that receptor molecule usage is strain-specific and/or cell type-dependent (Medigeshi *et al.*, 2008).

Entry of flavivirus particles into cells

Flaviviruses enter cells through clathrin-mediated endocytosis. Single-particle tracking analysis of DENV particles in living cells revealed that DENV particles diffuse along the cell surface towards a pre-existing clathrin-coated pit (van der Schaar *et al.*, 2008). This implies that virions roll over distinct attachment factors until they bind to the entry receptor localized to clathrin hotspots at the cell surface or that the initially formed virus-receptor complex is transported towards a pre-existing clathrin-coated pit. Subsequently, the clathrin-coated pit evolves and the invagination in the plasma membrane is closed by membrane scission mediated by dynamin to form a clathrin-coated vesicle. The clathrin-coated vesicle is transported away from the plasma membrane after which the clathrin coat is released from the vesicle. Real-time microscopy ana-



lysis showed that DENV particles remain associated with clathrin for approximately 80 seconds (van der Schaar *et al.*, 2008). Earlier evidence that flaviviruses utilize clathrin-mediated endocytosis for cell entry was obtained by ultrastructural studies showing the presence of Kunjin and YFV in coated pits (Ishak *et al.*, 1988; Ng & Lau, 1988). Furthermore, inhibition of WNV infection was observed in cells treated with chemical inhibitors like chlorpromazine (Nawa *et al.*, 2003) that prevent clathrin-coated pit formation and in cells expressing dominant-negative mutants of Eps15, a protein which is involved in clathrin-coated pit formation (Chu & Ng, 2004a; Chu *et al.*, 2006). The route of flavivirus cell entry appears to be dependent on the virus strain and the cell type as a recent report documented entry of DENV in mammalian cells independent of clathrin, caveolae and lipid rafts (Acosta *et al.*, 2009).

After clathrin-mediated uptake, the endocytic vesicle carrying the virus is delivered to early endosomes. Internalization of flavivirus particles occurs rapidly as a large fraction of WNV and DENV particles were observed to localize to early endosomes within 5 minutes post-entry (Chu & Ng, 2004a; van der Schaar *et al.*, 2008). Thereafter, the early endosome carrying the virus matures into a late endosome. For DENV, membrane fusion has been observed to occur primarily from within late endosomal compartments (van der Schaar *et al.*, 2008). Membrane fusion was detected on average at 10-13 minutes after initiation of infection. It is important to note that the subcellular compartment from which membrane fusion occurs is most likely dependent on the pH-dependent membrane fusion properties of the virus and may therefore vary between individual DENV strains (Krishnan *et al.*, 2007; van der Schaar *et al.*, 2008).

MOLECULAR MECHANISM OF MEMBRANE FUSION

Low-pH-induced conformational changes in the E glycoprotein

The low-pH environment within endosomes triggers a series of molecular events within the E glycoprotein leading to membrane fusion of the viral membrane with the endosomal membrane and subsequent release of the nucleocapsid into the cell cytosol. Protonation of one or more histidine residues has been postulated to trigger the conformational changes of the E protein (Harrison, 2008; Stiasny *et al.*, 2009). Indeed, studies on TBEV identified two conserved histidines (at position 146, 323) that may act as pH sensors and destabilize the DI-DIII interface (Fritz *et al.*, 2008). On the other hand, site-specific mutagenesis studies on WNV revealed that histidine residues are not required for the initiation of the conformational changes required for membrane fusion (Nelson *et al.*, 2009).

The conformational changes of the E glycoprotein that occur during the membrane fusion process of flaviviruses have been studied extensively (Mukhopadhyay *et al.*, 2005; Harrison, 2008; Stiasny *et al.*, 2009). The initial step in membrane fusion involves protonation-dependent disruption of the E protein rafts at the viral surface, including dissociation of E homodimers into monomers. This leads to the outward projection of DII and exposure of the fusion loop at the distal tip of DII to the target membrane. Subsequently, the E proteins insert their fusion loops into the outer leaflet of the membrane and three copies of E interact with one another via their fusion loops or DII domains to form an unstable trimer. The E trimers stabilize through additional interactions between the DI domains of the three E proteins (Liao *et al.*, 2010). Next, DIII is believed to fold back against the trimer to form a hairpin-like configuration. The

energy released by these conformational changes induces the formation of a hemifusion intermediate, in which the monolayers of the interacting membranes are merged while the inner membranes are still intact. Finally, a fusion pore is formed and after enlargement of the pore, the nucleocapsid is released into the cytosol.

Role of cholesterol in flavivirus membrane fusion

Besides the mildly acidic endosomal pH, it is the composition of the target membrane that plays an important role in the membrane fusion process of flaviviruses. In vitro studies have revealed that flaviviruses such as TBEV and WNV have the capacity to fuse with artificial receptor-free lipid membranes (liposomes) consisting of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) at low pH, albeit with low efficiency (Gollins & Porterfield, 1986; Corver *et al.*, 2000; Moesker *et al.*, 2010). Addition of cholesterol to target membranes was found to have a strong promoting effect on the membrane fusion capacity of TBEV and WNV(Gollins & Porterfield, 1986; Corver *et al.*, 2000; Stiasny *et al.*, 2003; Moesker *et al.*, 2010). Subsequent virus-liposome coflotation studies have indicated that cholesterol stimulates the low-pH-triggered interaction of the E glycoprotein with lipid membranes (Stiasny *et al.*, 2003; Umashankar *et al.*, 2008). The 3-hydroxyl group of cholesterol is important for this function (Stiasny *et al.*, 2003). Interestingly, and in contrast to alphaviruses, the E glycoprotein does not appear to directly interact with cholesterol in the target membrane (Umashankar *et al.*, 2008). These observations suggest that the promoting effect of cholesterol on membrane fusion is due to an overall change in the fluidity or physico-chemical properties of the target membrane. Although most studies were performed with TBEV and WNV several recent studies show that DENV also has the capacity to interact and fuse with artifical membranes consisting of PC, PE, sphingolipids and cholesterol (Poh *et al.*, 2009; Yu *et al.*, 2009; Schmidt *et al.*, 2010). Cholesterol also plays an important role in facilitating efficient cell entry of flaviviruses as viral infectivity was found to be significantly impaired in cholesterol-depleted cells (Medigeshi *et al.*, 2008; Das *et al.*, 2010; Puerta-Guardo *et al.*, 2010; Tani *et al.*, 2010).

Role of negatively charged lipids in dengue virus membrane fusion

A recent study from Zaitseva and coworkers showed that DENV may utilize anionic lipids as a cofactor during the low-pH-driven membrane fusion process of the virus (Zaitseva *et al.*, 2010). The investigation, by these authors, of the lipid dependence of DENV membrane fusion was triggered by the observation that DENV does not fuse with the plasma membrane of mammalian cells under low-pH conditions whereas efficient fusion occured with the plasma membrane of insect cells. A difference between these cell types is that insect cells have an unusually high concentration of anionic lipids in their plasma membrane. Accordingly, the authors found that addition of anionic lipids, such as bis(monoacylglycero)phosphate (BMP) and phosphatidylserine (PS), to the plasma membrane of mammalian cells facilitates low-pH-induced plasma membrane fusion of DENV. The role of anionic lipids in DENV membrane fusion was confirmed by in vitro virus-liposome fusion measurements, in which efficient membrane fusion was only observed using target membranes consisting of PC (70 mol%) and BMP or PS or phosphatidylglycerol (PG). Furthermore, anionic lipids were observed to act downstream of

the formation of a restricted hemifusion intermediate and likely promote the opening of the fusion pore. Interestingly, in mammalian cells, anionic lipids are enriched in late endosomal compartments, thus this observation may also explain why DENV fusion is primarily initiated from within these compartments (van der Schaar *et al.*, 2008).

PARTICLE MATURATION STATUS AND INFECTIVITY

Flavivirus-infected cells are known to secrete a mixture of mature, immature and partially mature particles. The prM quantity appears to be different between flaviviruses; whereas high numbers of prM-containing particles have been described for WNV and DENV (Randolph *et al.*, 1990; He *et al.*, 1995; Putnak *et al.*, 1996; Anderson *et al.*, 1997; Se-Thoe *et al.*, 1999; Zybert *et al.*, 2008; Cherrier *et al.*, 2009; Moesker *et al.*, 2010), low numbers were observed for TBEV (Heinz *et al.*, 1994). A recent study showed that as much as 40% of all extracellular DENV particles derived from C6/36 mosquito cells are partially immature (Junjhon *et al.*, 2010). Overall, this demonstrates that flavivirus maturation during virus egress is rather inefficient. Upon assembly of immature virions in the ER, all virus particles are transported to the Golgi apparatus (Deubel *et al.*, 1981; Hase *et al.*, 1987; Ng, 1987; Mackenzie & Westaway, 2001; Welsch *et al.*, 2009). Within the mildly acidic environment of the Golgi, the virion undergoes a global conformational change leading to dissociation of E/prM heterodimers and formation of 90 homodimers (Heinz *et al.*, 1994; Yu *et al.*, 2008; Yu *et al.*, 2009). This conformational change allows the host protease furin to cleave prM to M and a "pr" peptide (Wengler & Wengler, 1989; Heinz *et al.*, 1994; Yu *et al.*, 2008; Yu *et al.*, 2009). The pr peptide dissociates from the particle upon release of the virion to the extracellular milieu (Wengler & Wengler, 1989; Heinz *et al.*, 1994; Yu *et al.*, 2009; Zheng *et al.*, 2010). It appears that furin processing of prM is rather inefficient and flavivirus particles that contain uncleaved prM proteins will, after the release of the virion to the extracellular milieu, reorganize back to form prM/E heterodimers at the viral surface.

Numerous functional studies have shown that fully immature particles are noninfectious (Randolph *et al.*, 1990; Heinz *et al.*, 1994; Stadler *et al.*, 1997; Zybert *et al.*, 2008; Yu *et al.*, 2008; Yu *et al.*, 2009; Moesker *et al.*, 2010). However, we and others recently demonstrated that fully immature particles can be rendered infectious by antibodies (Dejnirattisai *et al.*, 2010; Rodenhuis-Zybert *et al.*, 2010a). We observed that the lack of infectivity of fully immature particles was found to be related to inefficient binding to the cell surface (Rodenhuis-Zybert *et al.*, 2010a). Upon cell entry, immature virions are efficiently processed by furin, the pr peptide presumably being released at the low-pH environment of endosomes (Zheng *et al.*, 2010). Whereas proteolytic cleavage of prM is a prerequisite for viral infectivity, multiple studies have shown that complete cleavage is not required for infectivity (Randolph *et al.*, 1990; Zybert *et al.*, 2008; Junjhon *et al.*, 2008; Nelson *et al.*, 2008). In partially mature particles, the mature aspect of the virion is most likely responsible for virus cell binding and entry after which the processing of prM may occur within the target cell and membrane fusion may be initiated. The threshold for viral infectivity in relation to the number of prM proteins present at the viral surface is as yet not understood.

ROLE OF ANTIBODIES IN CONTROLLING FLAVIVIRUS INFECTIVITY

The antibody response to flaviviruses is predominantly directed against the viral surface proteins E and prM, and to a lesser extent to the non-structural flavivirus protein NS1 (Shu *et al.*, 2000; Cardosa *et al.*, 2002; Libraty *et al.*, 2002; Gromowski & Barrett, 2007; Gromowski *et al.*, 2008; Lai *et al.*, 2008). Numerous functional studies revealed that antibodies against prM and E have the capacity to control the infectious properties of the virus(Kaufman *et al.*, 1987; Kaufman *et al.*, 1989; Halstead, 2003; Huang *et al.*, 2006; Pierson *et al.*, 2007; Rodenhuis-Zybert *et al.*, 2010a).

Antibody-mediated neutralization of infection

Antibodies can neutralize viral infection by interference with cell entry such as receptor attachment, virus internalization, and membrane fusion. Indeed, antibodies directed against the E glycoprotein have been shown to prevent attachment of the virus to the natural receptor and/or act downstream of virus-receptor interaction (Nybakken *et al.*, 2005; Thompson *et al.*, 2009; van der Schaar *et al.*, 2009). Importantly, blockade of virus-receptor interaction will not lead to neutralization of infection per se as virus-immune complexes can be internalized through interaction of the antibody with Fc receptors expressed on immune cells. Uptake of virus-immune complexes via the Fc receptor will result in the delivery of the virus to acidic endosomes, presumably in a manner very similar to uptake of virus particles after interaction with its natural receptor. It is therefore generally believed that potent neutralizing antibodies act downstream of virus-receptor interaction.

The most potent neutralizing antibodies are strain-specific and directed against DIII of the E glycoprotein(Oliphant *et al.*, 2006; Lisova *et al.*, 2007; Oliphant & Diamond, 2007; Rajamanonmani *et al.*, 2009). Domain III is well exposed on the surface of virions(Kuhn *et al.*, 2002). Antibodies directed against epitopes located within DIII were observed to neutralize infection at a low concentration(Pierson *et al.*, 2007; Pierson & Diamond, 2008). This indicates that only a small fraction of the DIII epitopes need to be occupied by antibodies to achieve neutralization. In contrast, antibodies directed against DI/II are highly cross-reactive and weakly neutralizing (Pierson *et al.*, 2007; Nelson *et al.*, 2008; Lai *et al.*, 2008; Pierson & Diamond, 2008). DI/II epitopes are poorly exposed at the surface of mature virions and were observed to require virtually complete occupancy by antibodies to reach the stoichiometric threshold required for neutralization (Pierson *et al.*, 2007; Pierson & Diamond, 2008). Some DI/II antibodies fail to neutralize infection even at conditions of antibody excess, indicating that not all DI/II epitopes can be bound by antibodies. This is presumably related to the structural organization of the E protein within the virion, as mature virions lack a true T = 3 symmetry (Kuhn *et al.*, 2002).

Antibody-mediated enhancement of infection

Antibodies directed against the E glycoprotein were observed to facilitate infection when the antibody occupancy level does not exceed the treshold required for virus neutralization (Halstead & O'Rourke, 1977; Pierson *et al.*, 2007). In this case, antibodies mediate efficient binding and entry of particles through FcR-mediated endocytosis. Upon delivery of the particles to acidic endosomes, the unoccupied E proteins are postulated to directly undergo the conformational changes that support membrane fusion. Alternatively, the mildly acidic pH within the endosomal compartment causes dissociation of the antibody from the particle thereby allowing membrane fusion and infection(van der Schaar *et al.*, 2009). Uptake of virions through FcR-mediated entry is not only postulated to increase the number of infected cells, it is also described to positively influence the number of virus particles produced per infected cell (Chareonsirisuthigul *et al.*, 2007; van der Schaar *et al.*, 2009). Antibodies directed against DI/II are proned to set the stage to antibody-dependent enhancement (ADE) of infection as only at very high antibody concentrations neutralization of infection is observed.

Next to E antibodies, antibodies against the prM protein have recently been shown to mediate ADE of infection (Randolph *et al.*, 1990; Huang *et al.*, 2005; Huang *et al.*, 2006; Rodenhuis-Zybert *et al.*, 2010a). prM antibodies were found to be highly cross-reactive and weakly neutralizing (Dejnirattisai *et al.*, 2010). Antibodies directed against the prM protein have been observed to render noninfectious fully immature virus particles almost as infectious as wt virus in a furin-dependent manner (as discussed above) (Rodenhuis-Zybert *et al.*, 2010a). Given the relative high number of immature particles present within wt preparations and the high number of prM antibodies produced during natural infection is it likely that prM antibodies and immature particles contribute to disease pathogenesis.

Taken together, under specific circumstances, antibodies may facilitate efficient cell entry leading to a higher number of circulating virions early in infection. The high infected cell mass subsequently results in a strong unbalanced immune response that leads to enhancement of disease (Rothman & Ennis, 1999).

ACKNOWLEDGEMENTS

This work was supported by the Pediatric Dengue Vaccine Initiative, and by the Dutch Organization for Scientific research (NWO-ALW).

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Characterization of the functional requirements of West Nile virus membrane fusion

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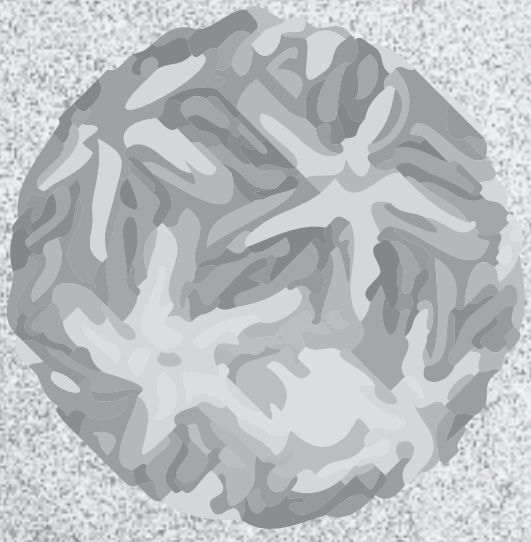
Journal of General Virology (2010), **91**: 389-393



Chapter 3

ABSTRACT

Flaviviruses infect their host cells by a membrane fusion reaction. In this study, we performed a functional analysis of the membrane fusion properties of West Nile virus (WNV) with liposomal target membranes. Membrane fusion was monitored continuously using a lipid mixing assay involving the fluorophore pyrene. Fusion of WNV with liposomes occurred on the timescale of seconds and was strictly dependent on mildly acidic pH. Optimal fusion kinetics were observed at pH 6.3, the threshold for fusion being pH 6.9. Preincubation of the virus alone at pH 6.3 resulted in a rapid loss of fusion capacity. WNV fusion activity is strongly promoted by the presence of cholesterol in the target membrane. Furthermore, we provide direct evidence that cleavage of prM to M is a requirement for fusion activity of WNV.

Functional requirements of West Nile virus fusion

West Nile virus (WNV) is a member of the genus *Flavivirus* which also includes dengue virus (DENV) and tick-borne encephalitis virus (TBEV). Flaviviruses infect cells via receptor-mediated endocytosis. In the acidic environment of the endosome, the viral envelope (E) glycoproteins undergo a series of orchestrated structural rearrangements, leading to fusion of the viral membrane with the endosomal membrane. Upon RNA replication and protein translation, prM/E-containing immature particles are formed by the budding of newly formed nucleocapsids into the endoplasmic reticulum (Lindenbach & Rice, 2001). These particles mature during transport through the Golgi and trans-Golgi network and shortly before the final release of the virions prM is cleaved to M by the host cell protease furin. Furin cleavage is not very efficient, as a mixture of prM-and M-containing particles are secreted from infected cells (Wengler & Wengler, 1989).

The flavivirus E-glycoprotein and alphavirus E1 glycoprotein are representatives of class II fusion proteins (Kielian & Rey, 2006). Class II fusion proteins share a similar architecture and undergo major structural rearrangements during their viral life cycle. For example, in the infected cell, class II fusion proteins are synthesized along with a companion protein (prM in flaviviruses and p62 or PE2 in alphaviruses) that stabilizes the fusion protein during transport through the secretory pathway. In addition, a series of conformational changes of the flavivirus E-and alphavirus E1-glycoproteins provide the driving force for membrane fusion. Exposure to low pH causes dissociation of the flavivirus E-homodimer or alphavirus E1–E2 heterodimer, which is followed by the formation of homotrimers (Sánchez-San Martín *et al.*, 2008). Despite the similarities in structure and overall mechanism of fusion, studies in liposomal model systems have revealed that the conditions that allow fusion may differ between flaviviruses and alphaviruses. For instance, alphavirus fusion is strictly dependent on the simultaneous presence of cholesterol (Chol) and sphingolipids in the target membrane (Nieva *et al.*, 1994; Smit *et al.*, 1999) while, in contrast, TBEV fusion does not appear to have such strict lipid requirements (Corver *et al.*, 2000; Stiasny *et al.*, 2003).

In this study, we investigated the functional requirements of WNV membrane fusion in a liposomal model system. Fusion of WNV with liposomes is efficient and occurs on the timescale of seconds. Optimal fusion kinetics were observed at pH 6.3, the threshold for fusion being pH 6.9. Furthermore, the presence of Chol in the target membranes significantly enhanced the membrane fusion potential of the virus. Finally, we demonstrate that maturation of WNV particles is required for membrane fusion activity.

To analyse the membrane fusion properties of WNV particles, we biosynthetically labelled the virus with the fluorescent probe pyrene, as described before for alphaviruses. Briefly, WNV strain NY385-99, a generous gift from Dr J. Goudsmit (Crucell B.V., The Netherlands) was added at an m.o.i. of 4 to baby hamster kidney cells (BHK-21) cultured beforehand in the presence of 15 µg 16-(1-pyrenyl)hexadecanoic acid (Invitrogen) ml^{-1} medium (Bron *et al.*, 1993; Smit *et al.*, 1999). At 24 h post-infection (p.i.), the virus particles were harvested and purified by ultracentrifugation (Smit *et al.*, 1999). The purity of the isolated fraction was checked by SDS-PAGE analysis and this revealed that the virus preparations were pure as only the viral proteins E, prM, C and M were observed (results not shown). Subsequently, the incorporation of pyrene into the viral membrane was evaluated by measuring the excimer-to-monomer (E/M) ratio in a Fluorolog 3-22 fluorimeter (BFi Optilas), essentially as described previously for TBEV (Corver *et al.*, 2000). We found an average E/M ratio of 0.29±0.14 (n=4), similar to the results obtained earlier with Semliki Forest virus (SFV) and TBEV (Bron *et al.*, 1993; Corver *et al.*, 2000). To ensure that pyrene-labelling did not affect the specific infectivity of the virus, we next determined the number of infectious units (IU) by titrating the virus on BHK-21 cells, and the number of physical particles by micro-Lowry protein determination. A theoretical amount of 2.26×10^{-17} g protein per virus particle was used to calculate the number of physical particles. The IU-to-particle ratio of unlabelled WNV was on average 1 : 850 (n=2), which is in agreement with earlier studies (Wengler & Wengler, 1989). Importantly, the average ratio for pyrene-labelled preparations was 1:550 (n=4), indicating that labelling had no effect on the infectivity of WNV.

Membrane fusion activity of pyrene-labelled WNV was measured in a liposomal model system. Upon fusion, the pyrene-labelled phospholipids will be diluted into the liposomes, resulting in a decrease of pyrene excimer fluorescence, which can be measured in an online fashion. Liposomes were prepared by a freeze–thaw extrusion procedure and consisted of a mixture of phosphatidylcholine (PC), phosphatidylethanolamine (PE), sphingomyelin (SPM) and cholesterol (Chol) at the indicated ratios, as described before (Smit *et al.*, 1999).

Figure 1(a) demonstrates that WNV fuses rapidly and efficiently with liposomes in a pH-dependent manner. Approximately 60 % of the particles fused with liposomes within 3 s after acidification to pH 6.3 (curve a). At pH 6.5, the initial rate of fusion was reduced compared with that at pH 6.3, but the extent remained close to 60 % (curve b). No membrane fusion was detected at pH 7.4 (curve c). A detailed characterization of the pH dependency is depicted in Figure 1(b). The fusion kinetics at physiologically relevant pH values below 6.3 were similar, both in terms of initial rate and final extent. A marked decrease in fusion activity was observed at pH 6.7, the threshold for fusion being pH 6.9. Furthermore, exposure of WNV to pH 6.3 in the absence of target membranes resulted in a rapid loss of fusion activity (Figure 1c). This shows that membrane fusion activation at low pH is of a transient nature, resulting in a rapid irreversible loss of membrane fusion capacity.

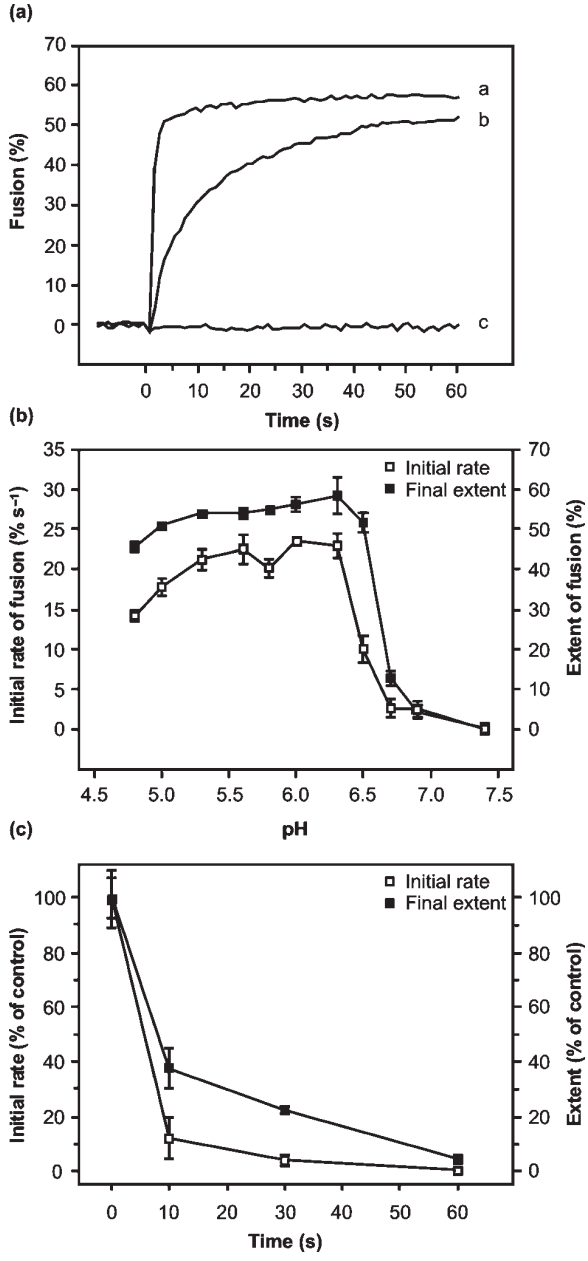


Figure 1. Low-pH-dependent fusion of WNV with liposomes. Pyrene-labelled WNV (1.5 µg protein; corresponds to $7x10^{10}$ particles) was mixed with liposomes (140 nmol phospholipid; corresponding to $3x10^{10}$ particles) at 37°C. Fusion was triggered by the addition of 0.1 M MES, 0.2 M acetic acid, pretitrated with NaOH to achieve the final desired pH. The fusion scale was calibrated such that 0 % fusion corresponded to the initial excimer fluorescence and 100 % fusion was obtained after the addition of 0.2 M octaethyleneglycol monododecylether. **(a)** Fusion of pyrene-labelled WNV with liposomes consisting of PC/PE/Chol (molar ratio 1 : 1 : 2). Curves: a, pH 6.3; b, pH 6.5; c, pH 7.4. **(b)** Kinetics of WNV fusion with liposomes as a function of the pH. The initial rates (open squares) were calculated from the tangent to the initial part of the curve and the final extents (solid squares) were determined at 60 s upon acidification. **(c)** Inactivation of WNV membrane fusion activity. Pyrene-labelled WNV was preincubated at pH 6.3 for the indicated periods of time, after which liposomes consisting of PC/PE/Chol (molar ratio 1 : 1 : 2) were added and fusion activity was measured. Fusion measurements were performed in triplicate. Error bars indicate SD.

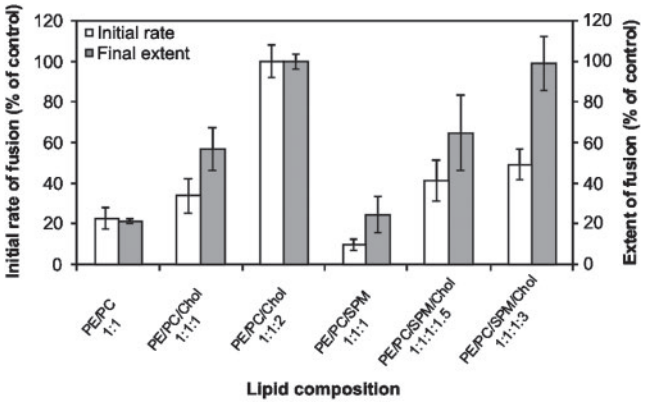


Figure 2. Influence of target membrane lipid composition on WNV membrane fusion. The fusion reaction was performed at pH 6.3, as described in the legend to Figure. 1. For clarity, the initial rate and final extent of fusion of the reaction with liposomes consisting of PC/ PE/Chol (molar ratio, 1 : 1 : 2) were set to 100 %. White bars, initial rate of fusion; grey bars, final extent of fusion. Data are shown as average values of at least two independent experiments performed in triplicate. Error bars indicate SD.

Subsequently, we studied the influence of the target membrane lipid composition on WNV fusion. Figure 2 shows that Chol has a strong promoting effect on membrane fusion activity. Analysis of the kinetics of fusion indicated that Chol mainly influenced the final extent of fusion as the initial rate (when related to the final extent reached) is constant in the presence and absence of Chol. The inclusion of SPM did not significantly enhance the fusion extent any further. Neither Chol nor SPM are essential for WNV membrane fusion, as target membranes consisting only of PC/PE supported fusion, albeit to a limited extent.

Earlier studies on TBEV and DENV have demonstrated that prM-containing particles are essentially non-infectious (Elshuber *et al.*, 2003; Zybert *et al.*, 2008). Furthermore, it has been shown that furin cleavage of prM to M is required for membrane fusion activity of TBEV on mosquito cells, as measured by a fusion-from-without assay (Guirakhoo *et al.*, 1991; Stadler *et al.*, 1997). Here, we used the liposomal model system to directly determine whether maturation is a prerequisite for fusion activity of WNV. To this end, we produced immature WNV on furin-deficient LoVo cells (Takahashi *et al.*, 1993; Zybert *et al.*, 2008). Briefly, LoVo cells were infected at an m.o.i. of 4. At 48 h p.i., the medium containing the virus particles was harvested and purified as described before (Zybert *et al.*, 2008). To determine the maturation status of LoVo-derived WNV, we produced ^{35}S-methionine-labelled virus using methods described before (Zybert *et al.*, 2008). Purified virus particles were subjected to SDS-PAGE analysis and the protein bands corresponding to the viral proteins were quantified using ImageQuant TL software (Molecular Dynamics). The percentage of immature particles in the preparation was determined by relating the intensity of prM and M to that of E, on the basis of the relative numbers of methionine residues in the distinct viral proteins. Figure 3(a) shows that LoVo-derived WNV is completely immature. Quantification of the protein bands revealed that the prM content of WNV grown on LoVo cells was 87±7 % compared with 30±11 % in BHK-21 cell-derived WNV. The relatively high number of prM-containing particles in wild type (wt) preparations is

in agreement with earlier studies (Wengler & Wengler, 1989). Subsequent determination of the specific infectivity revealed that immature WNV particles are essentially non-infectious as the p.f.u.-to-particle ratio of LoVo-derived WNV was approximately 50 000-fold lower compared with that of wt BHK-21-produced virus.

Next, we investigated whether the presence of prM obstructs membrane fusion activity, using a reverse variant of the pyrene fusion assay (Smit *et al.*, 1999). In this assay, a large excess of unlabelled WNV is incubated with pyrene-labelled liposomes and therefore it is well suited to determine the presence of fusion-active particles amongst a virus population. To measure fusion, liposomes with a diameter of 70 nm were used. Fusion of a 70 nm liposome with a viral lipid membrane of 40 nm in diameter (Kuhn *et al.*, 2002) will theoretically result in a ¼ increase in liposomal membrane surface area with a concomitant decrease of pyrene excimer fluorescence intensity of 25 %. Pyrene-labelled small unilamellar vesicles (pyrSUVs) were prepared as described previously (Smit *et al.*, 1999) and consisted of PC, 1-hexadecanoyl-2-(1-pyrenedecanoyl)-*sn*-glycero-3-phosphocholine (pyrPC), PE and Chol in molar ratios of 0.85 : 0.15 : 1 : 2, respectively. Figure 3(c) shows that pyrSUVs fused efficiently with wt WNV particles (curve a), with an average extent of pyrene excimer fluorescence decrease of 23 %. This corresponds to the theoretical value and suggests that all liposomes fused once with a virus particle under the conditions of the experiment. Again, fusion activity was strictly dependent on exposure of the virus-liposome mixture to mildly acidic pH. Immature particles failed to induce membrane fusion with liposomes at pH 6.3 (curve d), or lower (data not shown).

Subsequently, we investigated whether we could activate the membrane fusion potential by treatment of immature virus particles with exogenous furin. To this end, immature particles were incubated with furin (New England Biolabs) for 16 h at pH 6.0 and subsequently back-neutralized to pH 7.4. First, we determined by SDS-PAGE analysis, using ^{35}S-methionine-labelled virus, the ability of furin to cleave prM to M. Furin-treated immature WNV particles were completely mature, as the prM protein band was no longer visible on the gel (Figure 3a, lane 3). Likewise, titration on BHK-21 cells showed that the infectivity had increased by approximately 1000-fold (Figure 3b). Next, the fusion potential of these particles was assessed in a direct fusion measurement with pyrSUVs. Furin-treated immature WNV particles (curve b) fused efficiently with liposomes, which demonstrates that prM to M cleavage is required for membrane fusion activity.

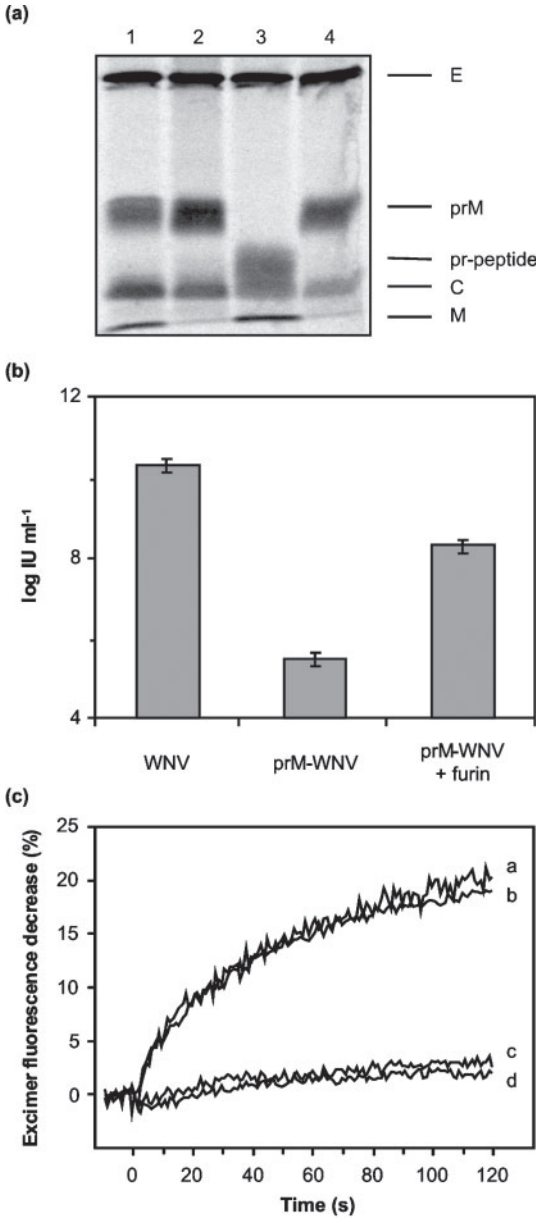


Figure 3. Influence of the maturation state on the fusogenic properties of WNV. (a) Protein composition. Lanes: 1, wt WNV; 2, immature WNV; 3, furin-treated immature WNV; 4, immature WNV treated for 16 h incubation at pH 6.0 in the absence of furin. Results shown are representative of three independent analyses. **(b)** Restoration of infectivity upon furin cleavage of immature WNV. Viral titres were determined on BHK-21 cells. Titres represent an average of three independent titrations. Error bars indicate SD. **(c)** Fusion activity of wt WNV, immature WNV and immature WNV upon furin cleavage. Virus (approx. 20 µg protein; corresponds to $1x10^{12}$ particles) was mixed with pyrene-labelled SUVs (0.5 mM phospholipid; corresponds to $1x10^{10}$ particles) consisting of PC/ pyrPC/PE/Chol (molar ratio 0.85 : 0.15 : 1 : 2) and fusion was measured. Curves: a, wt WNV at pH 6.3; b, furin-treated immature WNV at pH 6.3; c, wt WNV at pH 7.4; d, immature WNV at pH 6.3. Representative fusion curves of at least three experiments are shown.

Gollins and Porterfield (1986) showed before that WNV particles fuse with liposomes in a pH-dependent manner. In their work, fusion was measured quantitatively on the basis of content mixing between ^{3}H-uridine-labelled WNV with RNase-containing liposomes. Efficient RNA degradation was observed after 2 min incubation at low pH, indicating that membrane fusion occurs relatively quickly upon acidification. In this study, we obtained a detailed insight into the kinetics of membrane fusion as we measured fusion in a continuous fashion on the basis of lipid mixing. We observed that the majority of particles fused with liposomes within 3 s after exposure of the virus-liposome mixture to pH values ranging from pH 6.3 to 5.0. At pH 6.5, much lower fusion rates were observed even though maximum extent was reached. If we define the fusion curve as a cumulative plot of complete individual membrane fusion events, the lower rate of fusion suggests that the time from acidification to fusion and lipid mixing is variable under these conditions. This may reflect differences in the specific organization of viral glycoproteins on individual virions, as a mixture of mature and partially mature particles are suggested to be present in WNV preparations. At pH 6.7 slower kinetics and lower extents of fusion were observed, the threshold for fusion being pH 6.9. The kinetics of membrane fusion are similar to those described before for TBEV and faster compared with that of alphaviruses, which suggests that flaviviruses require a lower activation energy to drive the membrane fusion process (Bron *et al.*, 1993; Corver *et al.*, 2000).

The presence of Chol in target membranes strongly promotes membrane fusion activity of WNV. The highest extent of fusion was observed with liposomes containing 50 mol% Chol. The presence of Chol and SPM in target membranes is not required for membrane fusion activity, which suggests that, in general, flaviviruses exhibit a distinct lipid dependency compared with that of alphaviruses (Gollins & Porterfield, 1986; Corver *et al.*, 2000; Stiasny *et al.*, 2003; Umashankar *et al.*, 2008). Furthermore, our results demonstrate that the rate of fusion (related to the final extent reached) is similar in both the absence and presence of Chol, suggesting that Chol does not stimulate the fusion process itself. Instead, we propose that the presence of Chol facilitates a more stable initial interaction of WNV with target membranes. Indeed, coflotation studies showed that Chol strongly promotes low-pH triggered interaction of the fusion protein of alphaviruses (SFV) and flaviviruses (TBEV and DENV) with liposomes (Stiasny *et al.*, 2003; Umashankar *et al.*, 2008). In contrast to SFV E1, however, DENV E does not directly interact with Chol in the target membrane, which suggests that the observed promoting effect of Chol is due to the overall change of the lipid environment of the target membrane. Moreover, a recent study suggested that cholesterol-rich microdomains are involved in WNV uptake as it was observed that viral entry is inhibited in Chol-depleted cells (Medigeshi *et al.*, 2008). Our results show that WNV fusion proceeds efficiently in the absence of SPM, indicating that the presence of lipid rafts is not essential for WNV membrane fusion. However, we do not exclude the possibility that rafts are involved in the entry process of WNV.

It is generally accepted that the presence of prM in immature flavivirus particles obstructs viral infectivity (Elshuber *et al.*, 2003; Zybert *et al.*, 2008). Furthermore, studies on TBEV have revealed that cleavage of prM to M is important for the activation of the membrane fusion machinery, using a fusion-from-without assay in C6/36 cells (Guirakhoo *et al.*, 1991; Stadler *et al.*, 1997). In the present study, we analysed the fusion properties of immature and mature WNV

particles in an online fashion using a liposomal fusion assay. We show that immature particles lack the ability to induce membrane fusion. The infectious properties of WNV particles could be activated upon furin cleavage, after which a complete restoration of membrane fusion was observed. This proves that cleavage of prM to M is strictly required for expression of membrane fusion activity of WNV. Furthermore, our results indicate that immature particles, in contrast to wt virus preparations, are protected against low pH-mediated inactivation of membrane fusion activity. This substantiates the notion that upon cleavage of prM to M, the pr-peptide remains associated with the mature particle until it is returned to neutral pH, thus stabilizing the virus particle and preventing irreversible loss of membrane fusion that would otherwise occur under low pH conditions (Yu *et al.*, 2008).

ACKNOWLEDGEMENTS

This work was supported by the Pediatric Dengue Vaccine Initiative.

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A therapeutic antibody against West Nile virus neutralizes infection by blocking fusion within endosomes

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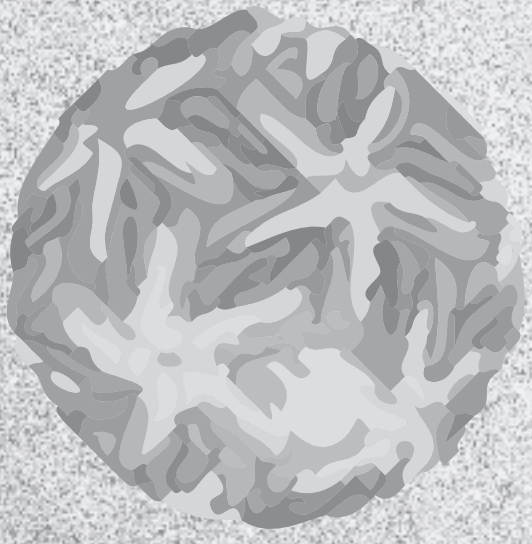
PLoS Pathogens (2009), **5**: e1000453



Chapter 4

ABSTRACT

Defining the precise cellular mechanisms of neutralization by potently inhibitory antibodies is important for understanding how the immune system successfully limits viral infections. We recently described a potently inhibitory monoclonal antibody (MAb E16) against the envelope (E) protein of West Nile virus (WNV) that neutralizes infection even after virus has spread to the central nervous system. Herein, we define its mechanism of inhibition. E16 blocks infection primarily at a post-attachment step as antibody-opsonized WNV enters permissive cells but cannot escape from endocytic compartments. These cellular experiments suggest that E16 blocks the acid-catalyzed fusion step that is required for nucleocapsid entry into the cytoplasm. Indeed, E16 directly inhibits fusion of WNV with liposomes. Additionally, low-pH exposure of E16-WNV complexes in the absence of target membranes did not fully inactivate infectious virus, further suggesting that E16 prevents a structural transition required for fusion. Thus, a strongly neutralizing anti-WNV MAb with therapeutic potential is potently inhibitory because it blocks viral fusion and thereby promotes clearance by delivering virus to the lysosome for destruction.

AUTHOR SUMMARY

Antibodies are essential components of the immune response against many pathogens, including viruses. A greater understanding of the mechanisms by which the most strongly inhibitory antibodies act may influence the design and production of novel vaccines or antibody-based therapies. Our group recently generated a highly inhibitory monoclonal antibody (E16) against the envelope protein of West Nile virus, which can abort infection in animals even after the virus has spread to the brain. In this paper, we define its mechanism of action. We show that E16 blocks infection by preventing West Nile virus from transiting from endosomes, an obligate step in the entry pathway of the viral lifecycle. Thus, a strongly inhibitory anti-West Nile virus antibody is highly neutralizing because it blocks fusion and delivers virus to the lysosome for destruction.

INTRODUCTION

Neutralizing antibodies can inhibit virus infection by impeding one of several critical steps of the virus lifecycle. These include blocking attachment to the cell surface, interaction with host factors required for internalization, and structural transitions on the virion that drive membrane fusion (reviewed in [1,2]). Antibodies can independently neutralize virus infection by promoting virus aggregation, destabilizing virion structure, and blocking budding or release from the cell surface (reviewed in [3]). Historically, many of the most potently neutralizing antibodies inhibit infection by interfering with required interactions between viruses and obligate cellular receptors (e.g., rhinovirus and ICAM-1, HIV and CD4 or CCR5, and poliovirus and CD155).

West Nile virus (WNV) is a mosquito-borne positive polarity RNA virus of the Flavivirus genus within the Flaviviridae family. Similar to other Flaviviruses, such as Dengue (DENV), yellow fever, and Japanese encephalitis viruses, WNV has an 11 kb RNA genome that encodes three structural (C, prM/M and E) and seven non-structural (NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5) proteins that are generated by cleavage from a single polyprotein [4,5]. WNV has spread globally and epidemic outbreaks of encephalitis now occur annually in the United States. Infection with WNV causes syndromes ranging from a mild febrile illness to severe neuroinvasive disease and death [6,7]. There is currently no approved vaccine or therapy for WNV infection.

Structural analysis of the WNV and DENV virions by cryo-electron microscopy [8,9] reveals a 500 Å mature virion with a smooth outer surface. The 180 copies of the E glycoproteins lay relatively flat along the virus surface as anti-parallel dimers in three distinct symmetry environments. Following exposure to low pH in the endosomal compartment, the E proteins rearrange from homodimers to homotrimers, exposing a fusion peptide, which interacts with the endosomal membrane and allows uncoating and nucleocapsid escape into the cytoplasm [10].

The atomic structure of the surface E glycoprotein has been defined by X-ray crystallography for DENV, WNV, and tick-borne encephalitis virus (TBEV) [11-15], revealing three conserved domains. Domain I (DI) is a 10-stranded β-barrel and forms the central structural architecture of the protein. Domain II (DII) consists of two extended loops projecting from DI and contains the putative fusion loop (residues 98-110), which participates in a type II fusion event [10,16,17]. In the mature virus, the fusion loop packs between two anti-parallel dimers and is solvent inaccessible, protecting the virus from premature fusion and inactivation. Domain III (DIII) is located on the opposite end of DI, forms a seven-stranded immunoglobulin-like fold, and has been suggested as a receptor binding site [18-20].

The humoral immune response controls WNV pathogenesis as mice lacking B cells are highly vulnerable to lethal infection [21]. During infection with flaviviruses, most neutralizing antibodies are directed against the E protein, although a subset binds the prM protein [22,23]. To better understand the structural basis of antibody protection against WNV, we recently generated a large panel of monoclonal antibodies (MAbs) against WNV E protein [24]. One antibody, E16, was observed to block WNV infection *in vitro* and *in vivo* and was effective as a post-exposure therapy even 5 days after infection [24,25]. Potent E16 neutralization occurs with strikingly low stoichiometric requirements, as a virion occupancy of ~25% is sufficient to inhibit infection [26]. Herein, we determine the mechanism by which this therapeutic MAb neutralizes WNV infection. E16 traffics with WNV particles into permissive target cells, and is strongly inhibitory because it blocks pH-dependent fusion, a critical step in the entry pathway of this virus.

RESULTS

MAb E16 does not block WNV entry

A common mechanism of antibody-mediated neutralization of viral infection is to prevent attachment and entry into target cells. Previously published studies suggested that E16 did not dramatically reduce WNV binding to Vero cells but instead inhibited at a post-attachment step [27]. To gain further insight as to how E16 inhibits infection, WNV was pre-incubated with Alexa-488 conjugated E16 or E53, a second inhibitory MAb that binds to the fusion loop in DII, prior to a cell binding assay at 4˚C. Subsequently, cells were washed at 4˚C, fixed and visualized by confocal microscopy. At 4˚C, enveloped viruses, including flaviviruses, remain on the cell surface and are not internalized [28-30]. As expected, in the absence of WNV, labeled E16 and E53 were not visualized on the surface or interior of cells (data not shown). When Alexa-488-E53-WNV complexes were added, no fluorescence signal was observed on the surface of Vero cells (Figure 1A, panels F and H), suggesting that E53, as hypothesized previously [27], primarily inhibits WNV attachment to Vero cells. Similar results were obtained with Alexa-488 conjugated E60, a MAb that binds to a similar epitope as E53 in DII (data not shown). In contrast, staining was apparent on the surface of cells incubated with labeled Alexa-488-E16-WNV complexes. Thus, despite saturating and neutralizing concentrations (100 μg/ml) of E16 MAb, WNV binding to Vero cells still occurred (Figure 1A, panels B and D). Analogous results were obtained with the strongly neutralizing DIII-specific E24 MAb (data not shown).

To determine if the E16 MAb restricted virus entry, Vero cells were warmed to 37˚C after MAb-WNV complex pre-binding at 4˚C, and again visualized by confocal microscopy. As anticipated, Alexa-488-E53-WNV complexes were not detected inside cells (Figure 1A, panels N and P). In contrast, Alexa-488-E16-WNV complexes readily entered cells and accumulated in acidic vesicles that were identified with a pH sensitive dye (Figure 1A, panels J and L). Even after several hours of incubation, E16-WNV complexes remained localized in these acidic cellular compartments (Figure 1B, panels B-D), whereas E53-WNV complexes were not detected within the cells (Figure 1B, panels F-H). In contrast, in the absence of neutralizing antibodies, WNV infection progresses rapidly as demonstrated by the accumulation of E protein in the cell over time (Figure S1).

E16 blocks infection in a manner analogous to inhibiting endosome acidification

Because E16-WNV complexes co-localized with an acidified intracellular compartment for several hours, we hypothesized that this MAb prevented virus fusion with endosomal membranes. Because WNV infection requires a pH-dependent structural rearrangement of E proteins for fusion, we evaluated whether concanamycin A1, a vacuolar-ATPase inhibitor [31], blocked WNV infection at a similar cellular stage as did E16. Vero cells were infected at a high multiplicity of infection (MOI) in the presence of 10 nM concanamycin A1 or humanized E16 (hu-E16, 100 μg/ml) or a media control for 3 h or 24 h at 37˚C. Cells were washed, fixed, and stained for WNV using an oligoclonal pool of mouse MAbs against the E protein. Samples treated with hu-E16 were also stained with an anti-human IgG secondary antibody to confirm that hu-E16 co-localized with the virus. In the absence of concanamycin A1 or hu-E16, infected Vero cells showed strong staining of E protein at 3 h that was markedly increased at 24 h (Figure S1). Treatment with 10 nM concanamycin A1 resulted in a punctate pattern of E protein staining

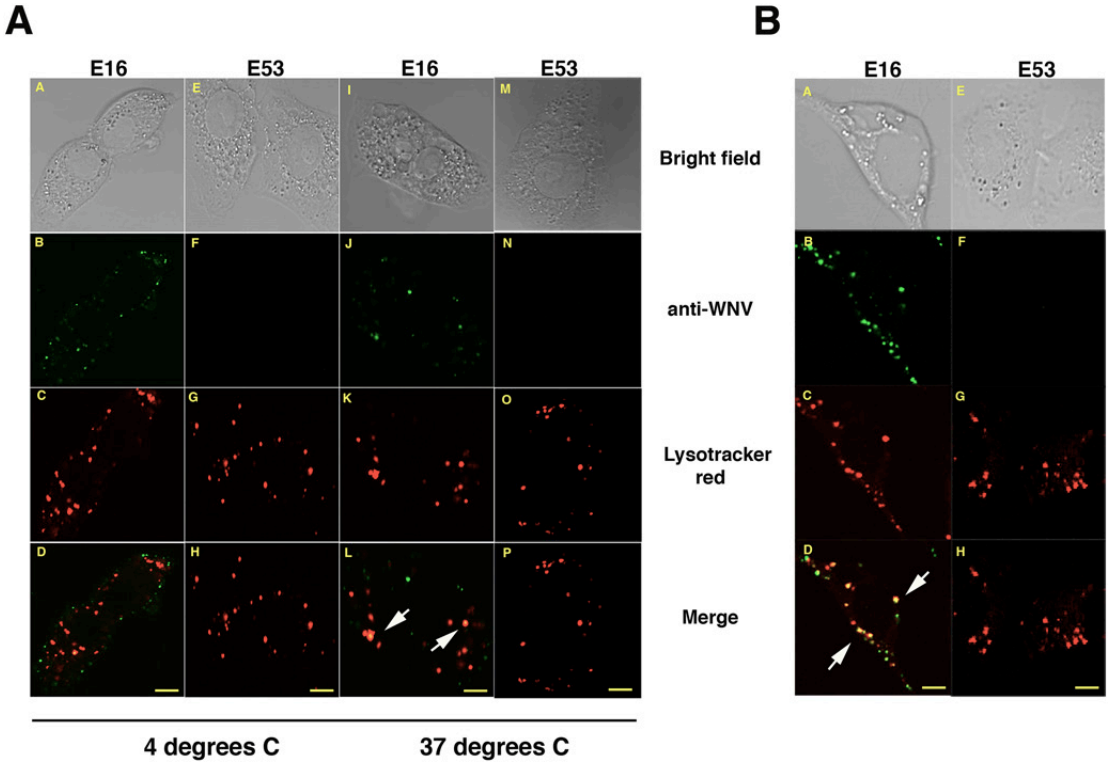


Figure 1. E16-opsonized WNV enters Vero cells. (A) Vero cells were incubated with WNV at an MOI of 100 in the presence or absence of 100 μg/ ml Alexa 488-E16 or E53 MAbs. Lysotracker red (50 nM) was added to the cells for the last 30 min of the incubation prior to paraformaldehyde fixation. Green staining indicates Alexa 488 conjugated anti-WNV MAbs (panels B, F, J, and N), red staining indicates lysotracker red dye (panels C, G, K, and O), and yellow staining represents co-localization as reflected by the merged images (panels D, H, L, and P). Cells were incubated for 2 h on ice, washed, fixed, and observed by confocal microscopy (panels A–H). Cells were shifted to 37˚C following the incubation on ice, fixed after 15 min and observed by confocal microscopy (panels I–P). White arrows indicate examples of co-localization of anti-WNV MAbs and lysotracker red dye. (B) Cells were infected for 3 h at 37˚C in the presence of 100 μg/ml E16 (B, D) or E53 (panels F and H) and lysotracker dye (panels C, D, G, and H) fixed, and analyzed by confocal microscopy. The scale bars represent 10 mm. doi:10.1371/journal.ppat.1000453.g001

at 3 and 24 h, suggesting that WNV localized to and likely remained sequestered in endocytic compartments (Figure 2, panels A and D). Analogous to treatment with concanamycin A1, hu-E16-opsonized WNV showed a similar staining pattern up to 24 hours after infection (Figure 2, panels B and E). As co-staining of oligoclonal mouse anti-E protein and hu-E16 was observed over time, it is likely that E16 was still bound to WNV, and these virus-MAb complexes accumulated in endosomal/lysosomal compartments (Figure 2, panels C and F). Of note, in Figure 2C, only a subset of the blue spots (which indicates the presence of the virion) co-stain with hu-E16. This is likely a sensitivity of detection issue as E16 neutralizes infection at both low (~25% or 30 copies per virion) and high occupancy [26]. Because of the high MOI used, some viruses will be more completely decorated (and thus fluorescent), whereas others will bind fewer antibodies yet still be neutralized. Virions that bind fewer E16 antibodies yet still are neutralized may co-stain less brightly in this microscopic assay.

E16 blocks fusion at the plasma membrane

The ability of E16 to block WNV egress from endosomes suggested that this MAb directly inhibited the pH-dependent fusion step. Initially, to test this, we used a surrogate plasma membrane fusion infection assay that has been validated for alphaviruses and flaviviruses [32,33]. Normally, flaviviruses enter cells via receptor-mediated endocytosis, with fusion occurring from within acidic endosomes [29,34,35]. However, flaviviruses also can be induced to fuse directly

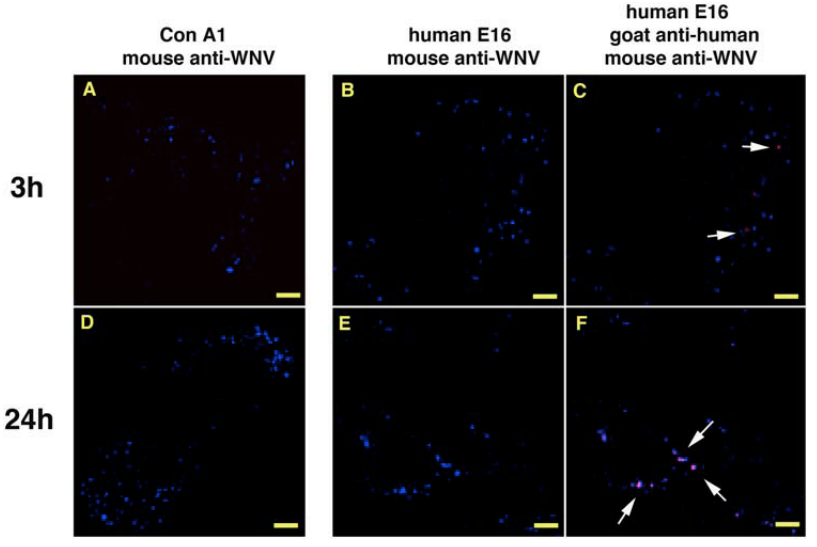


Figure 2. Blockade of endosomal acidification with concanamycin A1 mimics treatment with E16. Vero cells were infected at an MOI of 100 in the presence of 10 nM concanamycin A1 (panels A and D) or 100 μg/ml hE16 (panels B, C, E, and F) for 3 h (panels A–C) or 24 h (panels D–F) and then fixed. Cells were then stained with a pool of Alexa-488 conjugated mouse anti-E MAbs (blue; A-F) and Alexa-647-conjugated goat anti¬human IgG (red; C and F) as indicated. Cells were analyzed by confocal microscopy. The white arrows indicate co-localization of hu-E16 and the oligoclonal pool of mouse anti-E MAbs. Representative images are shown from one of two independent experiments. The images were analyzed using the LSM510 confocal microscopy software to assess the overlap in staining. Because of the overlay appearance, we converted the fluorescence images into blue (Alexa-488) and red (Alexa-647) colors for Figure display. The scale bars represent 10 mm. doi:10.1371/journal.ppat.1000453.g002

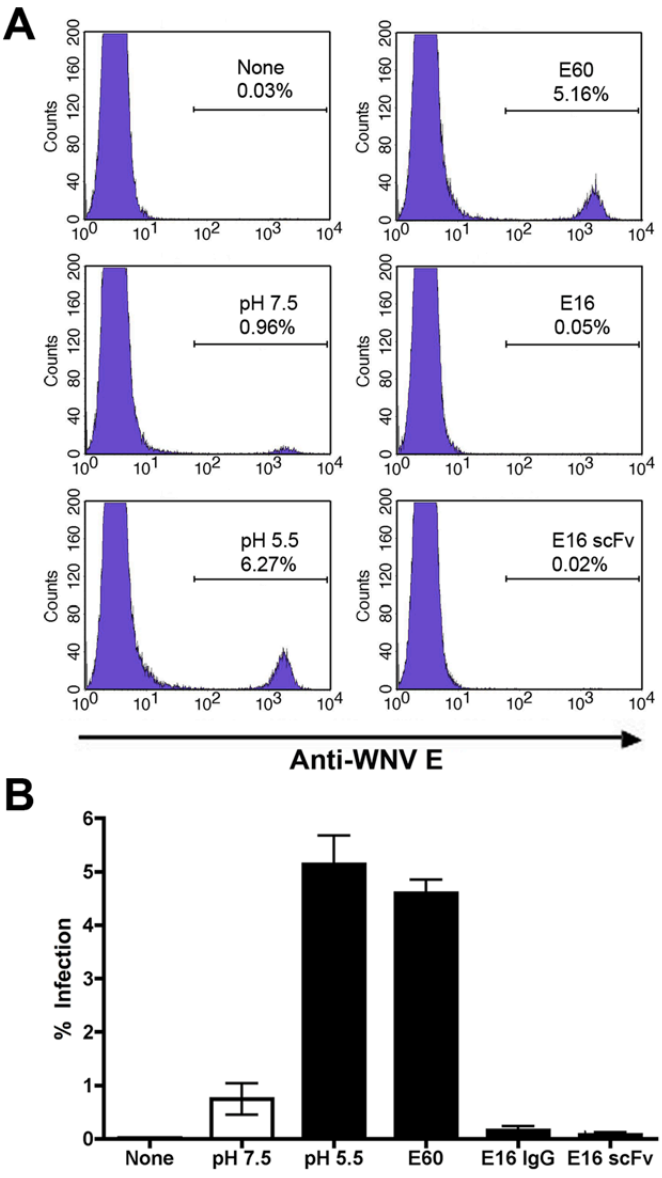


Figure 3. E16 scFv or IgG blocks infection in a plasma membrane fusion assay. WNV (10^6 PFU) was bound to Vero cells for 2 h on ice after pretreatment with 10 nM concanamycin A1. Subsequently, media, 100 μg/ml E16 IgG, E16 scFv or E60 IgG was added for 30 min on ice, and then the pH shifted at 37˚C to pH 7.5 or pH 5.5 for ~7 min. Cells were washed, the pH normalized, incubated at 37˚C for ~18 h, permeabilized and stained with an oligoclonal pool of anti-E MAbs. The level of infection was assessed by flow cytometry. (A) Representative flow cytometric histogram plots from each condition are shown. The plots are gated to show the percentage of cells that stained positive with an anti-WNV E MAb. The treatment and percentage of positive cells are shown in the top right corner of each plot. (B) The data averaged from three independent experiments is shown with error bars indicating standard deviations. Statistically significant differences between different experimental conditions are described in the text. doi:10.1371/journal.ppat.1000453.g003

with the plasma membrane, at low efficiency, when cell-bound virus is exposed to an acidic solution [32]. To assess the effects of E16 on virus-plasma membrane fusion, WNV was pre-bound to Vero cells at 4˚C, and subsequently incubated on ice with saturating concentrations of E16 IgG, E16 single chain Fv (scFv), E60 IgG, or no MAb. Cells were warmed to 37˚C in pH 5.5 media (or pH 7.5 media as a negative control) to induce virus-plasma membrane fusion and analyzed at 24 hours for level of infection by flow cytometry. In all experiments, 10 nM concanamycin A1 was added to inhibit infection via the canonical receptor-mediated endocytic pathway. As expected, in the absence of antibody, addition of media at neutral pH (7.5) did not promote productive infection (~0.7% WNV antigen$^+$ cells, Figure 3A and 3B). Exposure of cell bound WNV to media at pH 5.5 resulted in a ~7 fold increase in infection (~5.1% WNV

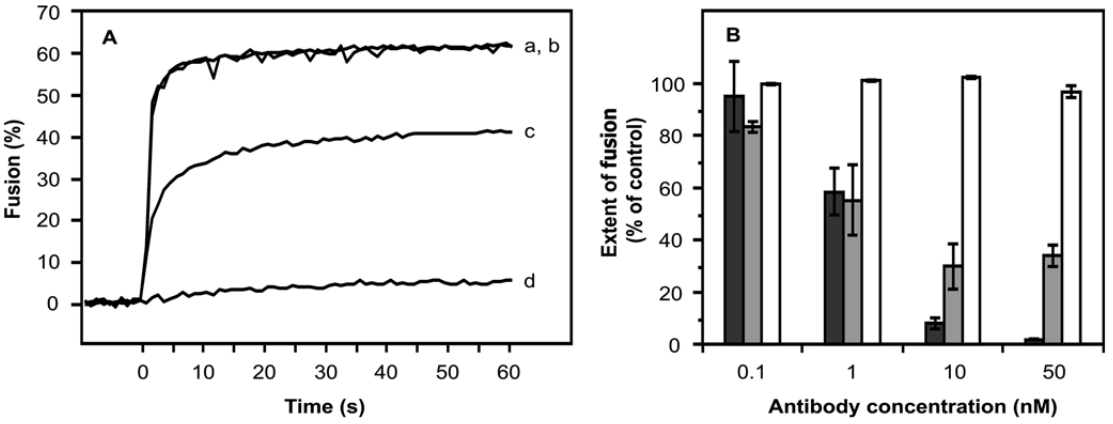


Figure 4. E16 blocks low pH-induced fusion of WNV with liposomes. Fusion of pyrene-labeled WNV with liposomes at pH 5.4. Fusion was measured in real-time as described in the Materials and Methods. (A) (a) no antibody; (b) 0.1 nM E16; (c) 1 nM E16; and (d) 50 nM E16. Representative viral fusion curves are from at least three independent experiments. (B) Effect of different concentrations of MAbs on WNV-liposome fusion. The WNV-liposome fusion profiles are shown as a percentage of the control (pH 5.4, without MAbs). Black bars, E16; gray bars, E60; and white bars, E111. Data are expressed as the mean of at least three independent experiments and the error bars indicate standard deviations. doi:10.1371/journal.ppat.1000453.g004

antigen$^+$ cells, P<0.0005, Figure 3A and 3B). The addition of E60 following viral attachment did not appreciably affect virus-plasma membrane fusion (P = 0.4), confirming earlier results that this MAb does not inhibit Vero cell infection at a post-attachment step [27]. In contrast, both E16 IgG and scFv efficiently blocked WNV-plasma membrane fusion (0.15% and 0.08% WNV antigen$^+$ cells, respectively; Figure 3A and 3B, P<0.0001).

E16 blocks pH-dependent fusion of WNV with liposomes

To confirm that E16 blocks membrane fusion of WNV, we evaluated the fusogenic properties of WNV in a model liposome system. To this end, WNV particles were metabolically labeled with pyrene hexadecanoic acid and purified by density gradient centrifugation. Subsequently, pyrene-labeled virions were pre-incubated with various concentrations of E16, E60 or E111 (a DIII-specific non-neutralizing control MAb [24]) and mixed with liposomes. The mixture was acidified to pH 5.4 and fusion was measured on-line in a fluorimeter at 37°C as a function of the decrease in pyrene excimer fluorescence. WNV fuses rapidly and efficiently with liposomes. In contrast, no membrane fusion activity was measured with saturating concentrations of E16 (Figure 4A). Inhibition of membrane fusion by E16 was dose-dependent as decreasing concentrations of E16 blocked fusion to a lesser degree (Figure 4A and 4B). E111 did not influence the membrane fusion properties of WNV as efficient fusion was measured at all antibody concentrations tested. MAb E60 was observed to induce a dose-dependent inhibition of membrane fusion activity, although a complete inhibition of fusion was not achieved (Figure 4B).

E16 Fab fragments prevent pH-dependent inactivation of WNV

Previous studies have shown that exposure of WNV or other flaviviruses to acidic (pH<6) media in the absence of target membranes results in E protein rearrangement, premature exposure of the fusion loop, virus aggregation, and rapid irreversible inactivation of fusion competence

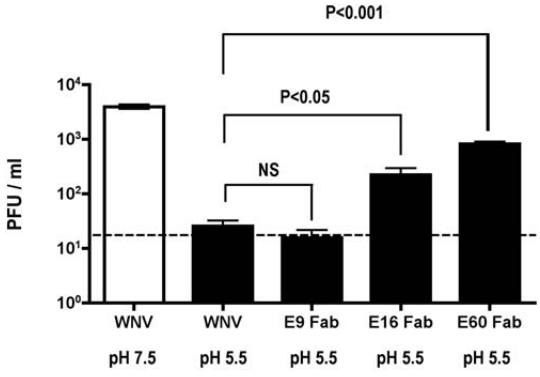


Figure 5. E16 Fab protects WNV from pH-induced inactivation in solution. WNV ($3x10^3$ PFU) was incubated alone in the presence of media, 100 mg/ml E16 Fab, E60 Fab or E9 Fab for 30 min on ice. The reaction was diluted 5-fold in media at pH 7.5 or pH 5.5 and incubated at 37˚C for 15 min, and then back-neutralized with a 25-fold excess of media at pH 7.5. This mixture was added to a monolayer of Vero cells prior to an overlay with 2% agarose. Three days later, plaques were fixed and scored. The dotted line represents the lower limit of detection the assay ($2x10^1$ PFU). Data is expressed as the mean of three separate experiments performed in duplicate. Statistical significance is indicated in the graph and was calculated using a two-tailed paired t test. doi:10.1371/journal.ppat.1000453.g005

[36–38]. We reasoned that if E16 neutralized WNV infection by directly blocking the pH-dependent fusion event it should prevent adventitious inactivation in solution after exposure to acidic pH. To test this, WNV ($3x10^3$ PFU) was pre-incubated with saturating (100 μg/ml) concentrations of E16, E60, or E9 (a DIII non-neutralizing MAb [24]) Fab fragments. Although the E60 MAb did not appear to enter cells or potently neutralize WNV infection [39], we included this fusion loop-specific Fab as a control because it partially inhibited pH-catalyzed virus fusion in the liposome assay. Excess buffered media at pH 7.5 or pH 5.5 was added to the virus-Fab complexes and incubated at 37˚C for 15 min. The solution was normalized after dilution with a 25-fold excess of pH 7.5 media and added to Vero cells for 1 h at 37˚C to allow infection as the monovalent Fab fragments detached. As expected, exposure to a pH 7.5 solution did not change WNV infectivity, as the monolayer contained ~$3.9x10^3$ PFU (Figure 5). In contrast, treatment with a pH 5.5 solution inactivated WNV and reduced infectivity ($P<0.0001$) below the limit of detection (~20 plaques). The E9 Fab failed to protect the virus from low pH inactivation, whereas neutralizing concentrations of E16 and E60 Fabs at pH 5.5 partially protected WNV from pH-induced inactivation as 2.2 and $8.2x10^2$ PFU were detected, respectively (Figure 5; $P<0.05$ and $P<0.0001$).

Because less infectious virus was detected with E16 compared to E60 treatment following pH normalization and dilution, we hypothesized that even a small number of bound E16 Fab could still inhibit infectivity since this MAb requires a low fractional occupancy for efficient neutralization [26]. Conversely, even detachment of a few E60 Fabs could significantly increase infectivity because virtually complete occupancy is required for neutralization by this MAb [40]. Experiments were repeated and excess recombinant E protein DIII (0.4 mg/ml) was added at the time of pH normalization and dilution to compete off additional bound E16 Fab. The addition of excess recombinant DIII further increased WNV infectivity by ~4 fold (data not shown), presumably by lowering the number of bound E16 Fab on some virions below the threshold for neutralization. Overall, these experiments show that saturating concentrations of both E16 and E60 Fabs at least, partially prevent irreversible pH-dependent inactivation of WNV in the absence of target membranes.

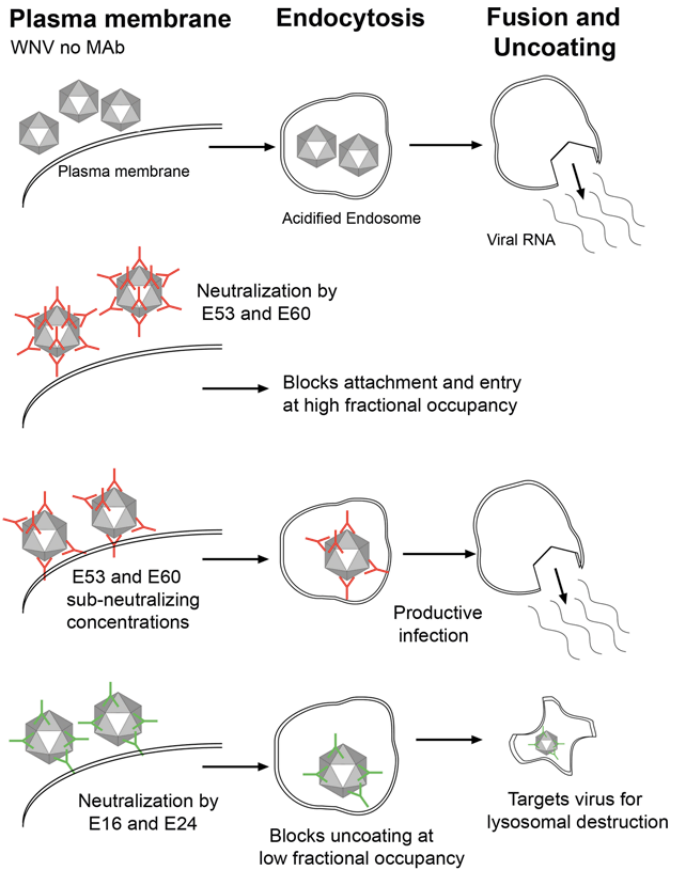


Figure 6. Model of anti–WNV MAb neutralization. The neutralization of WNV infection in Vero cells occurs by different mechanisms depending on the epitopes occupied by the MAbs. WNV infection in the absence of antibodies results in attachment, endocytosis, fusion, uncoating and release of the viral RNA into the cytoplasm. Vero cell infection in the presence of neutralizing concentrations of the fusion loop MAbs E53 or E60 results in a blockade in viral attachment. In contrast, infection in the presence of E53 or E60 at sub-neutralizing concentrations allows for efficient attachment, entry, fusion and infection. Infection in the presence of neutralizing concentrations of E16 or E24 (which require lower fractional occupancy for neutralization) results in relatively normal attachment and endocytosis. However, these MAbs inhibit fusion of the viral membrane with the endosomal membrane leading to subsequent targeting of the virus particles to the lysosome. doi:10.1371/journal.ppat.1000453.g006

DISCUSSION

Antibody neutralization is essential for protection against infection by many viruses. A greater understanding of the mechanism(s) by which the most strongly neutralizing antibodies act could facilitate strategies for generating targeted vaccines and immunotherapies. To establish the mechanism of action of E16, a strongly neutralizing anti-WNV MAb with therapeutic potential, we performed a series of cellular and biochemical experiments. Cell biology studies demonstrate that E16 blocks WNV infection at a post-entry stage by sequestering the virus in acidic compartments and preventing its egress into the cytoplasm. Biochemical experiments demonstrate that E16 neutralizes WNV by directly blocking the pH-dependent fusion process. Thus, the inhibitory activity of E16 against WNV *in vivo* is likely defined by its ability to block viral fusion and nucleocapsid penetration into the cytoplasm where replication occurs.

Analysis of the crystal structure of E16 Fab bound to WNV E conformations [10]. The biochemical data presented here protein led to a hypothesis that E16 blocked the structural demonstrating that E16 Fab blocks the pH-dependent inactivation rearrangement required for fusion at low pH [27]. Indeed, E16 of WNV in solution is consistent with a direct inhibition of the engages a large solvent-exposed surface of DIII, a domain that is structural transition of E protein that occurs during fusion. positioned distinctly in the pre-and post-fusion E protein

Nonetheless, definitive evidence of this structural mechanism awaits solution of the E16-WNV structure by cryo-electron microscopy in media at acidic pH.

In surface plasmon resonance (SPR) binding studies, E16 bound DIII of the WNV E protein with similar affinity across a range of pH values from pH 5 to pH 8 (B.S. Thompson, M.S. Diamond and D.H. Fremont, unpublished data). This explains why the binding and neutralizing activity of E16 is not altered as the virus-MAb complex transits through the endosomal compartments. Indeed, the confocal microscopy experiments showed co-localization of E16 and virus through acidic compartments into the lysosome. Our investigations with MAbs are consistent with an earlier study showing a strongly neutralizing polyclonal serum against WNV inhibited at a post-attachment step [41]; the authors of that study speculated but did not show that the most potently inhibitory antibodies block viral fusion. One reason why antibody blockade of fusion may be particularly potent *in vivo* for flaviviruses is because it acts downstream of an increasing number of cellular attachment factors (e.g., DC-SIGN, DC-SIGNR, heparin sulfate, Fc-γ receptors, and $\alpha_v\beta_3$ integrin [42-45]).

The confocal microscopy experiments also suggest that E16-opsonized WNV is retained in acidic compartments that are ultimately targeted for degradation. Antibodies like E16 that block fusion may be particularly potent at clearing viral infection *in vivo* because in addition to directly limiting transit to and replication in the cytoplasm they effectively convert permissive cells into ones that target virus for destruction. This feature of E16, along with its ability to disrupt transneuronal spread [46], high affinity, and capacity to neutralize at low virion occupancy [26], begins to explain its single-dose potent post-exposure therapeutic activity in animals [24,47].

The mechanistic analysis of E16 and WNV is supported by recent studies with MAbs against DIII of TBEV, some of which also blocked fusion of pyrene-labeled virus with liposomes [48]. Nonetheless, it remains unclear if the DIII MAbs against TBEV have equivalent neutralizing capacity and bind the same structural epitope as E16. The TBEV study also showed that DII-fusion loop MAbs were effective at blocking liposomal fusion. Although we also observed efficient dose-dependent inhibition of membrane fusion with E60, approximately one-third of the virus particles remained fusion competent even under conditions of antibody excess. This data is consistent with our observation that E53 and E60 are less strongly inhibitory MAbs against WNV [39] and that heterogeneity of WNV particles with respect to their state of maturation (mostly immature, partially mature, or fully mature) affects the ability of fusion loop MAbs to bind and neutralize infection [40]. As the fusion loop epitope is poorly accessible on the mature WNV virion [13,40,49], E53 and E60 MAbs require a relatively high fractional occupancy to inhibit infection [40]. Indeed, they may not achieve sufficient MAb concentration in the endosomes to neutralize by this mechanism. Instead, at least for Vero cells, our data with E53 and E60 suggests that antibodies of this class block at a proximal attachment step [27]. Based on these observations, we have developed a model for how the DII-fusion loop and DIII-lateral ridge MAbs neutralize WNV infection (Figure 6).

Blockade of viral fusion by antibodies or pharmacologic agents is usually considered as a therapeutic strategy for viruses that fuse with the plasma membrane. For example, enfuvirtide (Fuzeon™ or T-20 peptide) effectively inhibits entry of HIV at the plasma membrane of $CD4^+$ T cells by interfering with the requisite structural transition that brings viral and cell surfaces into proximity for fusion (reviewed in [50]). In contrast, there have been relatively few descriptions of antibodies that neutralize flaviviruses by blocking endosomal fusion. Butrapet et al described an anti-Japanese encephalitis virus antibody (MAb 503) that inhibited fusion-induced syncytia of infected insect cells and virus internalization into Vero cells. Although they concluded that this MAb functioned at a step post-attachment, they did not clearly demonstrate that it directly blocked fusion [51]. Similarly, the mechanism of action of the potently neutralizing anti-DENV2 MAb, 3H5-1 [52], has been speculated. Whereas He et al, showed that 3H5-1 blocked attachment of DENV2 to Vero cells [53], Se-Thoe et al, using LLC-MK2 cells, concluded that 3H5-1 primarily blocked the DENV2 fusion at the plasma membrane [54]. We recently localized the epitope of 3H5-1 of DENV2 to residues in the N-terminal region and FG loops of the lateral ridge of DIII, in an analogous position to that for E16 and WNV DIII [55]. Although further studies are necessary, based on structural localization and functional potency, we speculate that 3H5-1 and other strongly neutralizing DIII lateral ridge MAbs inhibit flavivirus infections, at least in part through similar fusion blocking mechanisms.

In summary, our experiments define the mechanism of action of a potently inhibitory therapeutic antibody against WNV. E16 prevents egress of WNV from endosomes, leading to retention in progressively acidic compartments and likely destruction in the lysosome. Vaccines that skew the immune response towards production of antiviral antibodies that inhibit fusion may improve protection against challenge. For highly promiscuous viruses like flaviviruses, targeting of the endosomal fusion step may be particularly relevant given the discovery of increasing numbers of distinct entry pathways on mammalian cells [42,43].

MATERIALS AND METHODS

Cell culture and propagation of WNV

Vero cells were used for confocal microscopy experiments, the plasma membrane fusion assay, and to titrate infectious virus by plaque assay. Vero cells were grown in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% FBS, 10 mM HEPES and 1% penicillin/streptomycin, as described [56]. WNV (strain 3000.0259, New York, 2000) [57] was propagated in C6/36 Aedes albopictus cells, aliquotted, and frozen at -80˚C.

Pyrene-labeled virus particles

Pyrene-labeled WNV was isolated from the medium of infected BHK21 cells that was cultured in the presence of 15 µg/ml of 16-(1-pyrenyl)-hexadecanoic acid (Invitrogen, Breda, The Netherlands), essentially as described before for alphaviruses [58,59]. BHK21 cells were infected at a MOI of 4. At 24 h post-infection, the supernatant was harvested and pyrene-labeled WNV particles were pelleted by ultracentrifugation (Beckman type 19 rotor; 15 hr at 48,500 x g at 4˚C). Subsequently, the virus particles were purified on an Optiprep (Axis-Shield, Oslo, Norway) density (15– 55% w/v) gradient by ultracentrifugation (Beckman SW41 rotor; 18 hr at 100,000 x g at 4˚C). The infectivity of the virus preparation was determined by titration on BHK21-15 cells. Protein concentration was determined by micro-Lowry analysis.

Preparation of liposomes

Large unilamellar vesicles were prepared by a freeze/thaw extrusion procedure as described [59]. Liposomes consisted of phosphatidylcholine (PC) from egg yolk, phosphatidylethanolamine (PE) prepared by transphosphatidylation of egg PC, and cholesterol in a molar ratio of 1:1:2. Liposomes were prepared with an average size of 200 nm. All lipids were obtained from Avanti Polar Lipids (Alabaster, AL).

Antibodies and flow cytometry

The anti-WNV antibodies E9, E16, E24, E53, E60, and E111 have been previously described [24,27,39]. Fab fragments were generated by papain digestion and purified by protein A affinity and size exclusion chromatography as described [27]. The generation and purification of the E16 scFv will be described in detail elsewhere (B. Kauffman, S. Johnson, D. Fremont, M. Diamond, and M. Rossmann, manuscript in preparation). Direct conjugation of MAbs to fluorochromes was performed using an Alexa Fluor™ 488 (or 647) MAb labeling kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Both anti-human and anti-mouse secondary antibodies conjugated to fluorochromes were purchased (Invitrogen) and used at a 1:200 dilution for confocal microscopy and flow cytometry. Flow cytometric analysis was performed using a BD FACS Calibur and BD Cellquest Pro™ software (Becton Dickinson, San Jose, CA).

Virus binding assays and confocal microscopic analysis

Vero cells were plated at ~7,500 cells/well in 8-well Lab-Tek chambered slides (Nunc, Rochester, NY) and incubated overnight. The cells were infected with WNV (MOI of 100) in the presence or absence of Alexa-488 conjugated antibodies at the indicated temperature and time, washed with PBS, and fixed with 2% paraformaldehyde in PBS for 30 min at room temperature. Acidified endosome and lysosome compartments were identified with Lysotracker red (Invitrogen) by adding the dye (50 nM) to the cells for the last 30 min of the incubation prior to fixation. To assess whether blockade of endosomal acidification mimics treatment with E16, Vero cells were infected at an MOI of 100 in the presence of 10 nM concanamycin A1 or 100 mg/ml hE16 for 3 h or 24 h, fixed with 2% paraformaldehyde,

and permeabilized with PBS supplemented with 0.1% saponin. Cells were stained with a pool of Alexa-488 conjugated mouse anti-E MAbs and in some experiments, Alexa-647-conjugated goat anti-human IgG. After extensive washing and fixation, cells were analyzed by confocal microscopy using a Zeiss LSM510 META Laser Scanning Confocal Microscope (Carl Zeiss Inc., Thornwood, NY) as described [60]. Images were analyzed using the LSM510 software suite and VolocityTM software package (Improvision Inc., Waltham, MA).

Plasma membrane fusion assay

The assay for plasma membrane fusion of flaviviruses has been described previously [32]. We adapted the protocol to test the effects of MAbs on WNV fusion at the plasma membrane. Briefly, Vero cells were plated in 12 well plates at $5x10^4$ cells per well and incubated for 24 h at 37˚C. The cells were then pre-incubated with 10 nM concanamycin A1 for 30 min. WNV (MOI of 100) was complexed with 100 µg/ml E16 IgG, E16 scFv, E60 IgG or control medium for 30 min at 4˚C and bound to Vero cells for 2 h on ice. Subsequently, cells were washed twice with iced PBS and pre-warmed DMEM (buffered to pH 5.5 or pH 7.5) was added at 37˚C for ~7 min. The cells were then washed with PBS and incubated for 24 h at 37˚C in DMEM containing 10 nM concanamycin A1, which blocks virus fusion after receptor mediated entry pathways. The cells were washed twice in PBS and fixed in PBS with 2% paraformaldehyde, permeabilized with 0.1% saponin and stained with an oligoclonal pool of Alexa Fluor-488-labeled anti-WNV MAbs. Samples were processed by flow cytometry and data was analyzed using the Cellquest Pro™ software.

pH inactivation assay in solution

WNV (~$3x10^3$ PFU) was incubated alone or with 100 µg/ml E16 Fab, E60 Fab or E9 Fab in DMEM at neutral pH for 30 min at 4˚C. The reactions were then diluted 5-fold in DMEM supplemented with 20 mM succinic acid (pH 5.5) or 20 mM HEPES (pH 7.5) and incubated at 37˚C for 15 min. Each reaction was subsequently neutralized by a 25-fold dilution in DMEM at pH 7.5 and added to a monolayer of Vero cells in a 6 well plate for 1 h at 37˚C. Following this incubation, the cells were overlaid with 2% low melting agarose and a standard plaque assay was performed. In some experiments, recombinant DIII (0.4 mg/ml) purified from E. coli [27] was added at the time of 25-fold dilution to compete bound Fabs.

WNV–liposome fusion assay

Fusion of pyrene-labeled WNV with PE/PC/cholesterol (molar ratio of 1:1:2) liposomes was monitored continuously in a Fluorolog 3–22 fluorometer (BFi Optilas, Alphen aan den Rijn, The Netherlands), at excitation and emission wavelengths of 345 nm and 480 nm. Pyrene-labeled WNV (0.35 µg protein; corresponds to $1.5x10^{10}$ particles) and an excess of liposomes (140 nmol phospholipid; corresponds to $3x10^{10}$ liposomes) was mixed in a final volume of 0.665 ml in 5 mM HEPES pH 7.4, 150 mM NaCl, and 0.1 mM EDTA. The content was stirred magnetically at 37˚C. At t = 0 sec, the pH of the medium was adjusted to 5.4 by addition of 35 µl 0.1 MES, 0.2 M acetic acid, pre-titrated with NaOH to achieve the final desired pH. The fusion scale was calibrated such that 0% fusion corresponded to the initial excimer fluorescence value. The 100% value was obtained through the addition of 35 µl 0.2 M octaethyleneglycol monododecyl ether (Fluka Chemie AG, Buchs, Switzerland) to achieve an infinite dilution of the probe. The extent of fusion was determined 60 seconds after acidification. To analyze the influence of E16, E60, and E111 on WNV fusion, pyrene-labeled WNV was incubated with increasing concentrations of MAbs for 1 hr at 20˚C prior to mixing with liposomes.

SUPPORTING INFORMATION

Figure S1 Cells were infected with WNV in the absence of MAbs for (A) 3, (B) 6, and (C) 24 hours as indicated, fixed, stained with an oligoclonal mixture of anti-E MAbs, and analyzed by confocal microscopy. Representative images are shown from one of at least four independent experiments. Found at: doi:10.1371/journal.ppat.1000453.s001 (3.22 MB TIF)

ACKNOWLEDGMENTS

The authors would like to thank S. Johnson of MacroGenics for providing some of the antibody reagents, T. Pierson for critical comments and thoughtful discussion, and Dr. Wandy Beatty for imaging expertise and advice.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: BST JMS JW MSD DHF. Performed the experiments: BST BJSM JMS. Analyzed the data: BST BJSM JMS JW MSD DHF. Contributed reagents/materials/analysis tools: MSD. Wrote the paper: BST BJSM JMS JW MSD DHF.

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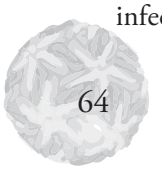

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Human monoclonal antibodies against West Nile virus induced by natural infection neutralize at a postattachment step

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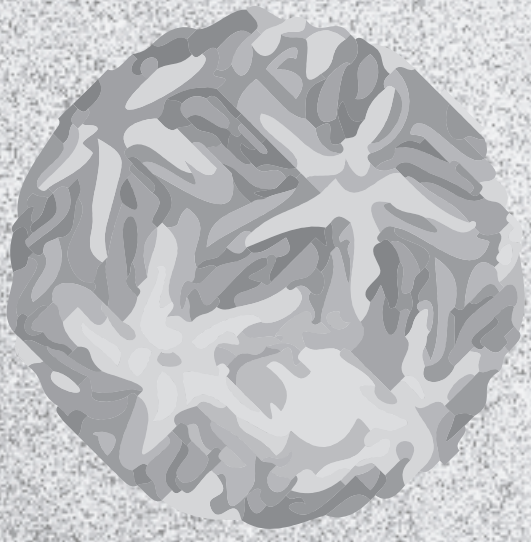
Journal of Virology (2009), **83**: 6494-6507



Chapter 5

ABSTRACT

West Nile virus (WNV) is a neurotropic flavivirus that is now a primary cause of epidemic encephalitis in North America. Studies of mice have demonstrated that the humoral immune response against WNV limits primary infection and protects against a secondary challenge. The most-potent neutralizing mouse monoclonal antibodies (MAbs) recognize an epitope on the lateral ridge of domain III (DIII-lr) of the envelope (E) protein. However, studies with serum from human patients show that antibodies against the DIII-lr epitope comprise, at best, a minor component of the human anti-WNV antibody response. Herein, we characterize in detail two WNV-specific human MAbs, CR4348 and CR4354, that were isolated from B-cell populations of convalescent patients. These MAbs strongly neutralize WNV infection of cultured cells, protect mice against lethal infection in vivo, and yet poorly recognize recombinant forms of the E protein. Instead, CR4348 and CR4354 bind determinants on intact WNV virions and subviral particles in a pH-sensitive manner, and neutralization is altered by mutations at the dimer interface in domain II and the hinge between domains I and II, respectively. CR4348 and CR4354 human MAbs neutralize infection at a postattachment step in the viral life cycle, likely by inhibiting acid-induced fusion within the endosome.

INTRODUCTION

West Nile encephalitis virus (WNV) is a positive-polarity, single-stranded RNA virus of the genus Flavivirus within the family Flaviviridae. Other members of this genus that cause significant human disease include dengue virus (DENV), St. Louis encephalitis virus, Japanese encephalitis virus (JEV), yellow fever virus, and tick-borne encephalitis virus (TBEV). Flaviviruses are translated as a single polypeptide, which is then cleaved by host and viral proteases into three structural (capsid [C], premembrane [prM], and envelope [E]) and seven nonstructural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) proteins (reviewed in references 42 and 43).

WNV cycles in nature between several species of birds and Culex mosquitoes, with humans and other mammals as dead-end hosts (25, 62). Infection causes syndromes ranging from a mild febrile illness to severe encephalitis and death (13, 72). WNV has spread globally and causes outbreaks with thousands of severe human cases annually in the United States. An age of greater than 55 years, a compromised immune status, and a CC5132 genotype have been associated with more-severe disease (15, 20). There is currently no approved vaccine or therapy for WNV infection.

The mature WNV virion has a ~500-Å diameter and consists of a single RNA genome surrounded by the capsid protein, a lipid bilayer, and a shell of the prM/M and E proteins (31, 55). X-ray crystallography studies have elucidated the three-domain structure of the flavivirus E protein (30, 48, 50, 58, 67). Domain I (DI) is a central, eight-stranded β-barrel, which contains the only N-linked glycosylation site in WNV E. Domain II (DII) is a long, finger-like protrusion from DI and contains the highly conserved fusion peptide at its distal end. Domain III (DIII) adopts an immunoglobulin-like fold at the opposite end of DI and is believed to contain a site for receptor attachment (6, 8, 40).

Within an infected cell, progeny WNV are assembled initially as immature particles. In immature virions, three pairs of E and prM interact as trimers and form 60 spiked projections with icosahedral symmetry (85, 86). Exposure to mildly acidic conditions in the trans-Golgi secretory pathway promotes virus maturation through a structural rearrangement of the E proteins and cleavage of prM to M by a furin-like protease (41, 83). Mature WNV virions are covered by 90 antiparallel E protein homodimers, which are arranged flat along the surface in a herringbone pattern with quasi-icosahedral symmetry (55).

Upon binding to poorly characterized cell surface receptors, internalization of WNV is believed to occur through receptor-mediated, clathrin-dependent endocytosis (1, 79, 80). After trafficking to Rab5-and/or Rab7-positive endosomes (38, 79), the mildly acidic pH within the lumen of the endosome induces structural alterations in the flavivirus E protein (7, 49), which includes changes in its oligomeric state (7, 49, 77). During this process, also known as type II fusion, the hydrophobic peptide on the fusion loop of DII of the E protein inserts into the endosomal membrane, thus physically joining the host and viral membranes, which allows the infectious RNA genome to enter the cytoplasm (32, 33).

Humoral immunity is an essential component of the protective host response against flaviviruses including WNV (reviewed in references 64 and 68). Studies by several groups have shown that the neutralization of WNV can occur after antibodies bind to a series of discrete epitopes on all three domains of the E protein (3, 12, 22, 59, 61, 71). To date, the most po-

tently neutralizing monoclonal antibodies (MAbs) localize to an epitope on the lateral ridge of DIII (DIII-lr). One well-characterized strongly neutralizing mouse MAb, E16, blocks infection primarily at a postattachment step (57) and requires the engagement of only a fraction of its epitopes on the surface of the virion (66). Studies of the human antibody response to WNV infection reveal that, in contrast to mice, antibodies that bind the DIII-lr epitope comprise a minor component of the neutralizing humoral response in most individuals (60).

In this study, we characterized two strongly neutralizing novel human MAbs (CR4348 and CR4354) that were selected from an antibody phage display library constructed from B cells of subjects that survived WNV infection (78). We demonstrate that both MAbs are WNV specific, bind weakly to recombinant or yeast surface-displayed E proteins, exhibit pH-sensitive binding to viral particles, and protect against lethal infection in mice. Our experiments suggest that these human MAbs map to distinct epitopes and neutralize infection at a postattachment stage, likely by inhibiting the acid-catalyzed viral fusion step.

MATERIALS AND METHODS

Preparation of virus, subviral particles, and pyrene-labeled virus.

WNV strain 3000.0259, which was isolated in New York in 2000 (16), was used to generate stocks of passage 2 ($4.2x10^7$ PFU/ml) and passage 3 ($2.0x10^7$ PFU/ml) virus after propagation in C6/36 *Aedes albopictus* cells. The propagations of wild-type and mutant WNV from the New York 1999 (NY99ic) infectious clone (5) are detailed below. WNV subviral particles (SVP) were generated after transfection of BHK21-15 cells with a pcDNA3.1 plasmid expressing premembrane (prM) and envelope (E) genes (36) from the NY99 WNV strain using FuGENE HD (Roche) according to the manufacturer's instructions. Supernatants containing SVP were collected 48 h after transfection, filtered through a 0.2-μm filter, and stored aliquoted at -80°C.

Labeling of WNV with the fluorescent probe pyrene was performed essentially as described previously for alphaviruses (73, 81). Briefly, BHK21-15 cells cultured in the presence of 15 μg/ml of 16-(1-pyrenyl)-hexadecanoic acid (Invitrogen) were infected with WNV at an multiplicity of infection of 4. At 24 h postinfection, the medium was harvested and clarified by low-speed centrifugation, and pyrene-labeled WNV particles were pelleted by ultracentrifugation in a Beckman type 19 rotor for 15 h at 48,500 x g at 4°C. The virus particles were further purified on an Optiprep (Axis-Shield) density (15 to 55%, wt/vol) gradient by ultracentrifugation in a Beckman SW41 rotor for 18 h at 100,000 x g at 4°C. The infectious titer was determined by 50% tissue culture infective dose analysis, and the protein concentration was measured by micro-Lowry analysis.

MAbs.

Unless otherwise specified, all antibodies used were protein A purified and of the human immunoglobulin G1 (IgG1) subclass. Humanized E16 (Hu- E16) was generated from a mouse MAb after genetic engineering as described previously (59). CR4348, CR4354, and CR4293 (anti-prM) were selected from single-chain variable-fragment (scFv) phage display libraries constructed from peripheral blood lymphocytes isolated from three human patients who survived neuroinvasive WNV disease (78). Construction of the libraries, selections with the libraries, and reformatting of scFv phage into full-length IgG1 molecules was previously described in detail (78). WNV E24 (mouse IgG2a) localizes to the DIII-lr epitope (59) and was purified by protein A affinity chromatography. Control non-WNV reactive anti-fluorescein isothiocyanate and anti-DENV1 E50 human IgG1 were gifts of S. Johnson (Macrogenics, Rockville, MD).

Neutralization assays.

(i) PRNT. In many experiments, the neutralizing activity of MAbs was determined using a 50% plaque reduction neutralization test ($PRNT_{50}$) analysis. Briefly, serially diluted MAbs were mixed 1:1 with 10^2 PFU of WNV in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS) (Omega Scientific) and incubated for 1 h at 37°C. The WNV-MAb mixture was then added to individual wells of a six-well tissue culture plate in duplicate or triplicate with either Vero or BHK21-15 cells. Viral adsorption proceeded for one hour at 37°C, followed by an overlay with 1% low-melt agarose (SeaPlaque) in α-modified Eagle medium and 4% FBS. After solidification, plaques were visualized 3 to 4 days later following fixation with 2 ml of 10% formaldehyde, removal of agarose plugs, and staining with 1% (wt/vol) crystal violet in 20% (vol/vol) ethanol. Plaques were counted and then normalized to the average of data from six control wells in which WNV was mixed with DMEM containing 10% FBS and no antibody.

(ii) Pre- and postattachment neutralization assays. To assay for postattachment neutralization, a PRNT assay was completed essentially as described above with the following modifications. All solutions and Vero cells were prechilled to 4°C, and 10^2 PFU of WNV were then added to each well of cells, and viral adsorption was allowed for 1 h at 4°C. Wells were then washed three times with medium, and MAb at the specified concentrations was added. Virion-antibody complexes were allowed to form for 1 h at 4°C, followed by three washes with chilled medium. Cells were then warmed to 37°C, and the PRNT was completed as described above. In parallel, a PRNT with all cells and solutions at 4°C was performed in which MAb and WNV were mixed for 1 h at 4°C prior to addition to cells (preattachment assay).

(iii) RVP assay. WNV and mutant reporter virus particles (RVP) were generated as described previously (65, 66). Separate plasmids expressing the wildtype prM-E genes and the capsid (C) gene of WNV were transfected into a BHK cell that stably propagates a WNV replicon expressing green fluorescent protein. In some experiments, the prM-E plasmid was mutated using the QuikChange site-directed mutagenesis kit (Stratagene) to introduce specific amino acid substitutions. In other experiments, RVP were produced from 293T cells at various stages of maturation (immature, partially mature, or fully mature) according to previously published protocols (56). Supernatants containing RVP were harvested 48 h after transfection, filtered through a 0.2-μm filter, and stored aliquoted at -80°C. RVP were incubated with serial dilutions of MAb under conditions of antibody excess at room temperature (RT) for 1 h. Subsequently, MAb-RVP mixtures were added to Raji-DCSIGNR cells, which stably express the DC-SIGNR attachment factor (14), and were incubated at 37°C for 48 h. Infected cells were assayed for green fluorescent protein expression using a BD FACSArray flow cytometer. Alternatively, human CD32A (Fc-γRIIA)-expressing K562 cells were used to assay for antibody-dependent enhancement of infection.

Yeast surface display of WNV E proteins.

The generation of *Saccharomyces cerevisiae* cells that express the WNV E protein ectodomain (amino acid residues 1 to 415) or DIII (residues 296 to 415) was described previously (59). Yeast cells expressing WNV E or DIII were washed in a solution containing phosphatebuffered saline (PBS), 2% bovine serum albumin, and 0.025% NaN_3; incubated with primary MAbs (50 μg/ml) for 30 min on ice; washed three times; mixed with a 1:500 dilution of Alexa Fluor 647-conjugated goat anti-human antibody (Molecular Probes); washed again; and processed using a BD FACSArray flow cytometer.

Antigen capture and solid-phase ELISA.

Nunc MaxiSorp polystyrene 96-well plates were coated either overnight at 4°C with murine DIII-lr MAb (10 μg/ml) or for 1 h at 37°C with the soluble recombinant WNV E protein ectodomain (10 μg/ml), generated as described previously (57), in a pH 9.3 carbonate buffer. Plates were washed three times in enzyme-linked immunosorbent assay (ELISA) wash buffer (PBS with 0.02% Tween 20) and blocked for 1 h at 37°C with ELISA block buffer (PBS, 2% bovine serum albumin, and 0.02% Tween 20). SVP or WNV infectious virions (wild type or mutant) were captured on plates coated with murine DIII-lr MAb for 1 h at RT. Subsequently, plates were rinsed five times in wash buffer and then incubated with anti-WNV or control human IgG1 (10 μg/ml in block buffer) in triplicate for 1 h at RT. Plates were washed five times and then incubated with biotinylated rabbit anti-human IgG antibody (1:1250 dilution; Southern Biotech) for 1 h at RT in blocking buffer. Plates were washed again five times and then sequentially incubated with 2 μg/ml of horseradish peroxidase-conjugated streptavidin (Vector Laboratories) and tetramethylbenzidine substrate (Dako). The reaction was stopped with the addition of 2 N H_2SO_4 to the medium, and emission (450 nm) was read using an iMark microplate reader (Bio-Rad). In the pH-dependent ELISA, plates were washed four times with wash buffer and once with the indicated pH buffer (150 mM NaCl, 0.05% Tween 20, and 50 mM MES [morpholineethanesulfonic acid] [pH 6.0]) or wash buffer (pH 7.4) after trapping of SVP. The plate was incubated for 30 min at RT with the indicated pH buffer and then washed five times in wash buffer followed by normal completion of the capture ELISA protocol.

Western blots.

The recombinant WNV E protein, SVP, or infectious WNV was diluted in 2 x sodium dodecyl sulfate loading buffer with or without β-mercaptoethanol (5%, vol/vol) and incubated at RT or 95°C for 10 min as indicated. Samples were loaded into wells of a NuPAGE (Invitrogen) 4 to 12% Bis-Trisgradient gel and electrophoresed. Protein was transferred onto a polyvinylidene difluoride transfer membrane using the iBlot system (Invitrogen). Membranes were rinsed in PBS–0.05% Tween 20 (wash buffer) with gentle shaking for 10 min at RT and then blocked overnight with block buffer (5% dry milk in wash buffer) with shaking at RT. After five 10-min washes, membranes were stained with primary human MAb (1 μg/ml diluted in block buffer with normal goat serum added at a 1:250 dilution) for 1 h at RT. After five additional washes, membranes were incubated with horseradish peroxidase-conjugated goat anti-human antibody (diluted 1:5,000 in block buffer; Sigma) for 1 h. Membranes were then washed five times for 10 min in wash buffer and developed using ECL reagent (Amersham).

Generation of neutralization escape mutants.

WNV was incubated with 25 μg/ml of CR4348 or CR4354 for 1 h at RT in DMEM. The mixture was added to Vero cells in a six-well plate at a multiplicity of infection of 1. After infection for 2 h at 37°C, wells were washed three times with DMEM, and fresh medium containing 25 μg/ml of MAb was added. Virus growth under antibody selection was allowed for 48 h at 37°C. At each passage, half of the supernatant was mixed 1:1 with 50 μg/ml of MAb for 1 h. The remaining half of the supernatant was aliquoted and stored at -80°C. After three passages under MAb selection, virus-containing supernatants were tested by PRNT for escape from neutralization by CR4348 or CR4354. After confirmation of the escape phenotype, an aliquot of the supernatant was used in a Vero cell plaque assay under MAb selection. Plaques were visualized by overlaying with neutral red, and sterile glass Pasteur pipettes were used to isolate resistant virus from single plaques. Plaquepurified virus was amplified under MAb selection (25 μg/ml) overnight at 37°C. Vero cells were scraped from wells, and total cellular RNA was isolated using an RNeasy kit (Qiagen). cDNA was amplified using a reverse primer (2501R [5'- TGCCGGCTGATGTCTATGG-3']) in the WNV NS1 gene and served as a template for PCR amplification of the prM and E genes using forward (454F [5'-AGCGTAG-

GAGCAGTTACCC-3']) and reverse (2501R) primers. The prM and E genes were then directly sequenced from gel-purified PCR products, and the neutralization escape mutant sequence was compared to the sequence of the laboratory stock WNV that was passaged and plaque purified in parallel in the absence of MAb selection.

WNV infectious cDNA clone and mutant generation.

The two-plasmid WNVNY99 cDNA clone (36) was used to create wild-type and mutant infectious WNV. Single amino acid substitutions were introduced into plasmid pWNAB by site-directed mutagenesis. Wild-type and mutant plasmid pWNAB (encoding nucleotides 1 to 2495 of the WNV genome) and wild-type plasmid pWNCG (encoding nucleotides 2495 to 11029) were grown in SURE-2 supercompetent Escherichia coli cells (Stratagene) at RT. Each plasmid was digested with XbaI and NgoMIV restriction endonucleases, and the resultant ~5.2- and 8-kb fragments of pWNAB and pWNCG, respectively, were gel purified and ligated with T4 DNA ligase (Invitrogen) at 4°C overnight. The reaction mixtures were then heat inactivated, digested with XbaI to linearize the DNA, treated with proteinase K, extracted twice with phenol and chloroform, and precipitated with ethanol at -20°C overnight. All DNA was used as a template for in vitro DNA-dependent RNA transcription with the AmpliScribe T7 kit (Epicentre) with the addition of an $m^7G(5')ppp(5')A$ cap analog (New England Biolabs). Transcription reactions were run at 37°C for 5 h, and the reaction mixture was then electroporated (three pulses at 850 V, 25 µF, and $\infty\Omega$) into BHK21-15 cells. Cells were added to a T75 tissue culture flask in DMEM with 10% FBS and observed for the onset of cytopathic effects. Once cytopathic effects were observed, the viruscontaining supernatant was collected, cellular debris was pelleted, and supernatant aliquots were frozen at -80°C. The cells remaining in the T75 flask were harvested and used as a source of viral RNA to confirm that the desired mutant sequence was retained.

Structural analysis.

The coordinates for the WNV E protein (RCSB accession number 2HG0) were divided by domains and fit onto the cryo-electron microscopy structure for mature DENV (RCSB accession number 1K4R) using CCP4MG to create a model of the WNV E protein dimer. Distances were calculated and figures were prepared using PyMol (http://www.pymol.org).

Mouse experiments.

Mouse studies were approved and performed according to the guidelines of the Washington University School of Medicine Animal Safety Committee. Three- to four-week-old outbred NIH Swiss mice (Harlan) were infected by intraperitoneal injection with WNV-NY99ic diluted in Hanks' balanced salt solution containing 1% heat-inactivated FBS. For antibody protection studies, 1 day prior to infection, mice were treated by intraperitoneal injection with 50 µg of the indicated MAb or vehicle control diluted in 100 l PBS. Mice were monitored daily for 21 days for mortality and were euthanized when moribund. In some experiments, passive transfer of MAbs was performed with 5-week-old C57BL/6 mice (Jackson Laboratories) as described previously (59).

Fusion assay.

The fusion of pyrene-labeled WNV with liposomes was monitored continuously in a Fluorolog 3-22 fluorometer (BFi Optilas) at excitation and emission wavelengths of 345 nm and 475 nm, respectively. Liposomes (large unilamellar vesicles with a diameter of ~200 nm) were prepared by a freeze-thaw extrusion procedure as described previously (73). Liposomes consisted of a mixture of phosphatidylcholine from egg yolk, phosphatidylethanolamine prepared by the transphosphatidylation of egg phosphatidylcholine, and cholesterol in a molar ratio of 1:1:2. The lipids were obtained from Avanti Polar Lipids. The concentration of phospholipids was determined by phosphate analysis.

Pyrene-labeled WNV (1.24 μg of total viral protein) and liposomes (final concentration, 200 μM phospholipid) were mixed in a final volume of 665 μl in a solution containing 5 mM HEPES, 150 mM NaCl, and 0.1 mM EDTA (pH 7.4) with continuous stirring in a temperature-controlled cuvette at 37°C. At 0 s, the medium was acidified by the addition of 35 μl of a solution containing 0.1 MES and 0.2 M acetic acid, which was pretitrated with NaOH to achieve the final desired pH. The fusion scale was calibrated such that 0% fusion corresponded to the initial excimer fluorescence value. The 100% value was obtained through the addition of 35 μl 0.4 M octaethyleneglycol monododecyl ether (Fluka Chemie AG) to achieve an infinite dilution of the probe. The extent of fusion was determined 60 s after acidification. To determine the influence of the MAbs on membrane fusion, pyrene-labeled WNV was incubated with different concentrations of MAbs for 1 h at RT prior to mixing with liposomes.

Statistical analysis.

All data were analyzed using Prism software (GraphPad, San Diego, CA). Kaplan-Meier survival curves were analyzed by the log-rank test. For neutralization assays, an unpaired Student's t test was used to determine significance. For ELISA data, a paired Student's t test was used to determine significance.

RESULTS

Characterization of strongly neutralizing human MAbs.

Previous studies that mapped the epitope specificity of inhibitory antibodies in convalescent-phase human serum samples suggested that only a fraction of WNV-infected patients developed antibodies against the strongly neutralizing epitope on DIII-lr of the E protein (60). Consistent with this, few DIII-specific human MAbs were isolated from phage display scFv libraries from infected patients (78), and none were selected from very large libraries of pooled sera from uninfected donors (22). As human convalescent-phase serum retains strong neutralizing activity, we hypothesized that antibodies with distinct specificities must contribute to the inhibitory activity.

Antibody-phage display libraries constructed from peripheral blood lymphocytes of three convalescent patients after WNV infection were screened for reactivity on WNV antigen as described previously (78). All selected monoclonal phages specifically bound a preparation of inactivated WNV and immobilized prM-E-containing SVP. SVP, in addition to containing prM/M proteins, display 60 E protein homodimers in a lipid bilayer (18), whereas virions have 90 E protein homodimers in a distinct icosahedral arrangement (39, 55). Two MAbs, CR4348 and CR4354, which demonstrated strong neutralizing activity in pilot functional assays, tested negative for binding to the recombinant E protein by ELISA (data not shown); in contrast, other neutralizing human MAbs that were identified in the screen and previously characterized (e.g., CR4374) (60, 78) readily bound the recombinant E protein.

In both the PRNT and WNV RVP assay, CR4348 ($PRNT_{50}$ = 536 ng/ml; RVP 50% effective concentration [EC_{50}] = 146 ng/ml) and CR4354 ($PRNT_{50}$ = 88 ng/ml; RVP EC_{50} = 26 ng/ml) inhibited infection strongly albeit slightly less than that achieved with a humanized version of a therapeutic WNV type-specific mouse MAb, E16 (Hu-E16), that maps to the DIII-lr epitope (59) (Fig. 1A and B). CR4348 and CR4354 appeared to be distinct from Hu-E16, as they did not efficiently recognize the ectodomain of the E protein when expressed as a purified protein or displayed on the surface of yeast cells (Fig. 2A and B). These neutralizing

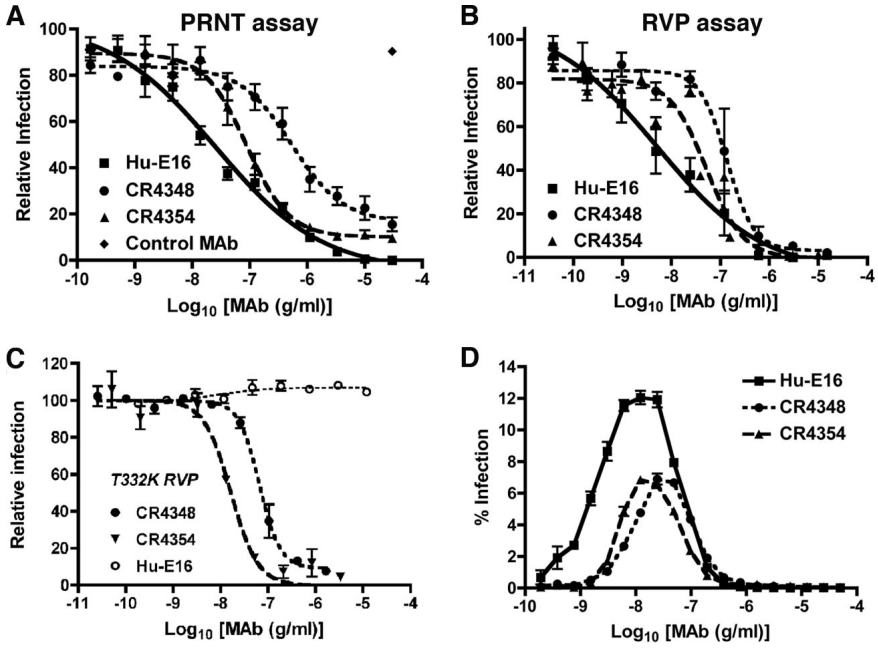


Figure 1. Neutralization and enhancement of human MAbs. (A) $PRNT_{50}$. Hu-E16, CR4348, CR4354, and an isotype control MAb (DENV1- E50) were tested for neutralization activity by standard PRNT on BHK21-15 cells. The data shown are combined results of data for two independent experiments performed in triplicate. The data are normalized to data from six control wells in each experiment with no MAb. (B and C) RVP neutralization assay. Hu-E16, CR4348, and CR4354 were incubated with RVP prior to infection of Raji-DCSIGNR cells. RVP were prepared normally (B) or with the T332K mutation in the E protein (C), which abrogates neutralization by virtually all DIII-lr MAbs. Data shown are representative of data from three independent experiments performed in duplicate. Error bars represent standard deviations, and lines represent curve fits generated by nonlinear regression analysis. (D) Hu-E16, CR4348, and CR4354 were tested for their abilities to enhance WNV RVP infection of human CD32 (Fc-γRIIA)-expressing K562 cells. Data shown are representative of data from at least two experiments performed in triplicate. Error bars represent standard deviations.

human MAbs also efficiently neutralized variant WNV RVP composed of E proteins encoding a T332K mutation that abrogates the neutralization of virtually all DIII-lr MAbs (Fig. 1C) (3, 59). Additionally, both human MAbs likely do not recognize linear epitopes, as they did not identify prM, M, or E by Western blotting under reducing or nonreducing conditions (Fig. 2C).

Because CR4348 and CR4354 neutralized WNV but did not appear to recognize the recombinant E protein, we speculated that they might inhibit infection by binding to an epitope on prM that was not be detected by Western blotting. To test this, we took advantage of a series of WNV RVP preparations that differ with respect to the efficiencies of the maturation process; similar preparations were used previously to define the differential neutralizing activity of anti-WNV MAbs generated in mice (56). WNV RVP were produced as fully mature (in cells overexpressing the furin protease), partially and heterogeneously mature (standard conditions), or largely immature (in the presence of NH_4Cl, which inhibits furin cleavage) particles. These three types of RVP differ in their content of prM, ranging from virtually none (mature) to >95% (immature). CR4348 and CR4354 neutralized prM-containing or -absent particles equivalently (Fig. 3), eliminating the pr peptide as part of the epitope for either MAb.

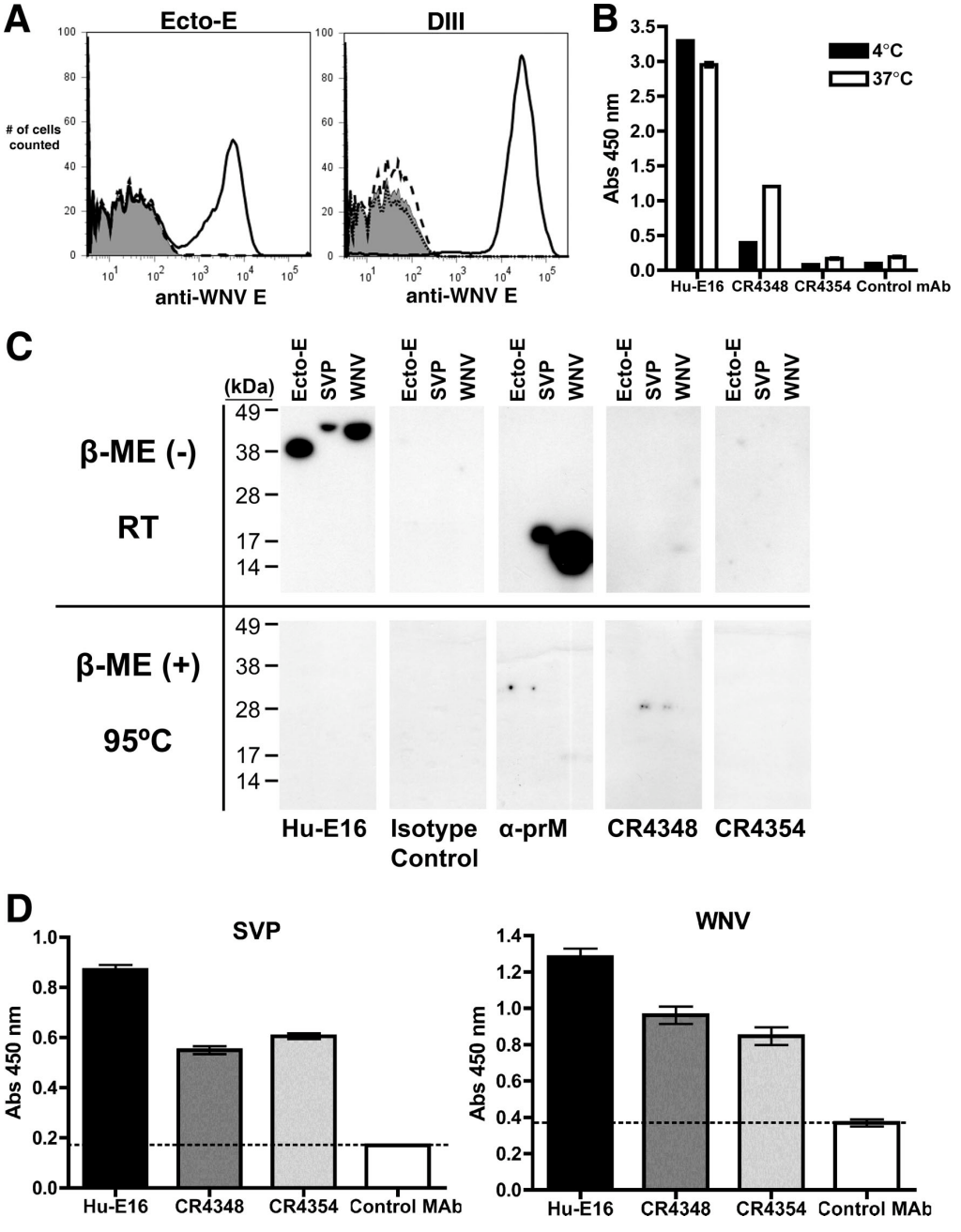


Figure 2. Binding of human MAbs to recombinant protein, SVP, and virions. (A) Yeast display assay. The WNV E protein ectodomain (Ecto-E) (amino acids 1 to 415) (left) or DIII alone (amino acids 296 to 415) (right) was expressed on the surface of yeast cells; stained with CR4348 (dotted lines), CR4354 (dashed lines), Hu-E16 (solid lines), or irrelevant human IgG1 (filled area) MAbs; and detected by flow cytometry. Data from one representative experiment of three are shown. (B) ELISA. The ectodomain (amino acids 1 to 415) of the E protein was adsorbed onto microtiter plates for 1 h at 37°C or overnight at 4°C. After blocking, wells were incubated with the indicated MAbs, and ELISA was performed as detailed in Materials and Methods. Data shown are representative of data from three experiments performed in triplicate, with error bars representing standard deviations. Abs, absor-

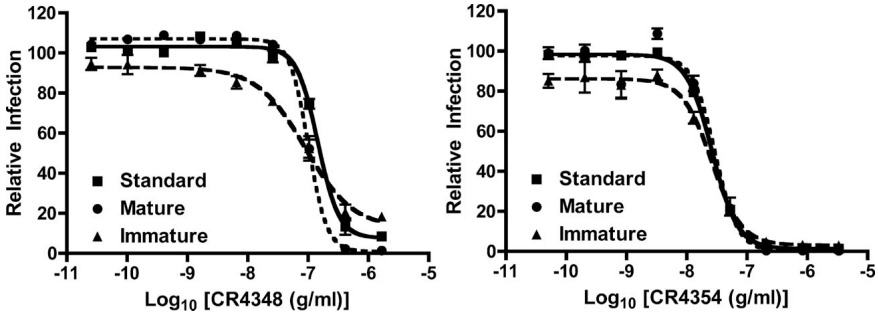


Figure 3. Effect of virus maturation state on neutralization by CR4348 and CR4354. CR4348 (left) and CR4354 (right) were incubated with MAbs prior to infection of Raji-DCSIGNR cells. RVP were prepared normally (mixture of mature, immature, and partially mature particles), in the presence of NH_4Cl (immature), or in cells overexpressing furin protease (mature) to create virions of different maturation states. These virions were incubated with MAbs prior to infection of Raji-DCSIGNR cells. Data shown are combined results from three independent experiments performed in duplicate.

An alternative possibility was that CR4348 and CR4354 do not bind a viral protein but instead inhibit WNV infection by binding a host cell surface receptor and blocking attachment and/or entry. However, if the neutralizing MAbs CR4348 and CR4354 recognized a structural protein on the virion, they should enhance infection in Fc-γ receptor-expressing cells when the stoichiometry of binding falls below the neutralization threshold (51, 66). The addition of subneutralizing concentrations of CR4348 and CR4354 to K562 cells that display human CD32 (Fc-γRIIA) enhanced infection in a manner that was analogous to that observed with Hu-E16 (Fig. 1D). This experiment suggests that CR4348 and CR4354 recognized a protein on the surface of the infectious virion.

To corroborate these findings, we used a capture ELISA to measure the direct binding of CR4348 and CR4354 to SVP and infectious WNV virions. Notably, CR4348 and CR4354 bound to both SVP and infectious virus ($P \leq 0.002$) (Fig. 2D). However, CR4348 and CR4354 did not bind to DENV particles, consistent with their inability to neutralize these viruses in a plaque reduction assay (data not shown). Thus, biochemical and functional analyses suggested that CR4348 and CR4354 neutralize WNV by virtue of their ability to bind an epitope on one of the viral structural proteins (M or E) that requires a specific oligomeric arrangement present on virions or SVP.

Neutralization by CR4348 and CR4354 occurs primarily at a postattachment step.

Antibody neutralization of enveloped viruses may occur by inhibiting receptor attachment, internalization, and/or endosomal fusion (63, 88). To determine the stage of the viral entry pathway at which CR4348 and CR4354 inhibit infection, we performed pre-and postattachment neutralization assays (11, 29, 57). CR4348 and CR4354 were incubated with WNV before

bance. (C) Western blot. The recombinant E protein ectodomain (~39 kDa), SVP (glycosylated full-length E protein) (~44 kDa), or WNV virus (unglycosylated full-length E protein) (~43 kDa) was assessed for binding by the indicated human MAbs to the E or prM (~15-kDa) protein. Samples were prepared in sodium dodecyl sulfate sample buffer with or without heating to 95°C and with or without 5% (vol/vol) β-mercaptoethanol (β-ME). (D) A capture ELISA was used to detect binding of MAbs to WNV SVP (left) and virions (right). Microtiter plates were coated with murine E16, incubated with SVP or virus, and detected with the indicated human IgG1 MAbs. Dashed lines indicate the background of the assay with an isotype control MAb.

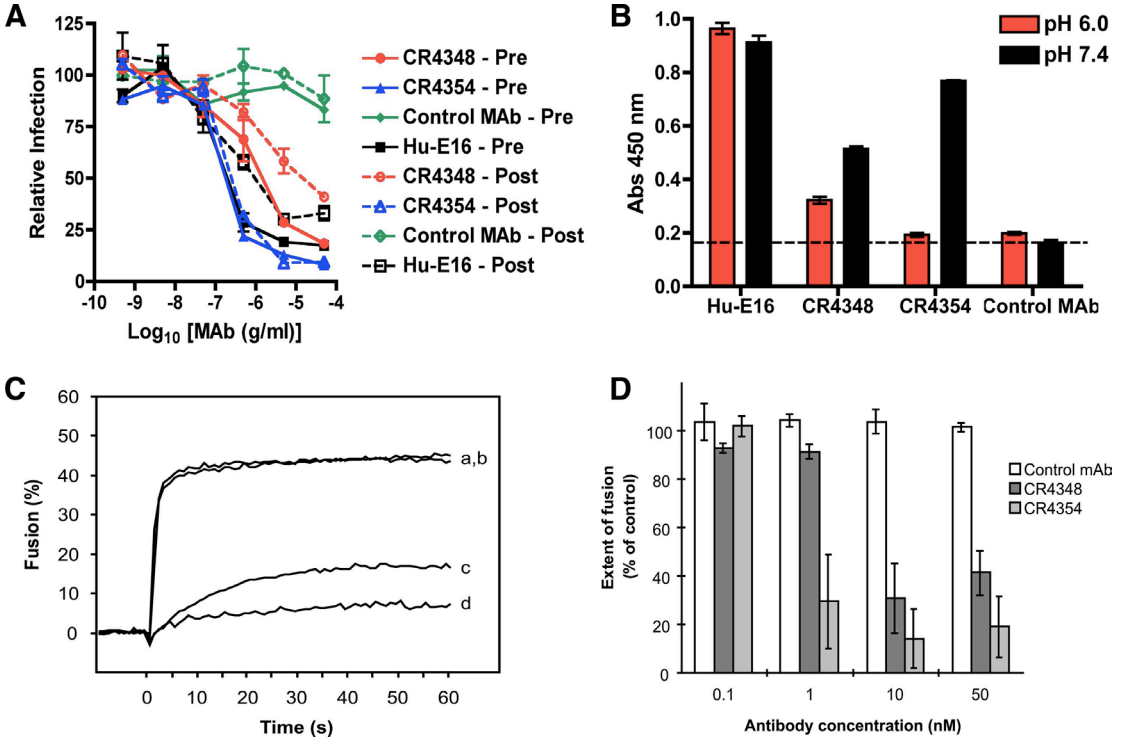


Figure 4. Mechanism of WNV neutralization by CR4348 and CR4354. (A) Pre- and postattachment inhibition assays. To determine whether the MAbs neutralize WNV infection after cellular attachment, Vero cells were prechilled to 4°C, and 10^2 PFU of WNV were added to each well for 1 h at 4°C. After extensive washing at 4°C, the MAbs were added for 1 h at 4°C, and the PRNT protocol was then completed (dashed lines) (Post). In comparison, a standard preincubation PRNT with all steps performed at 4°C is shown for reference. In this case, virus and MAb were incubated together for 1 h at 4°C, prior to addition to cells (solid lines) (Pre). Data shown are representative of data from three experiments performed in duplicate, with error bars representing standard deviations. (B) pH sensitivity of MAb binding to SVP. The capture ELISA protocol was modified such that the pH was changed (pH 6.0 or 7.4) for 30 min immediately before captured SVP were detected by the indicated human IgG1 MAbs. Data from one representative experiment of three performed in triplicate are shown, with error bars representing standard deviations. Dashed lines indicate the background of the assay with an isotype control MAb. Abs, absorbance. (C and D) Fusion of pyrene-labeled WNV with liposomes. (C) Pyrene-labeled WNV was incubated with or without 50 nM of the indicated MAbs for 1 h at room temperature prior to mixing with liposomes and acidification at pH 6.3. Fusion was measured online, as described in Materials and Methods. Representative fusion data for at least three independent experiments are shown. Curve a, no antibody; curve b, 50 nM isotype-matched control; curve c, 50 nM CR4348; curve d, 50 nM CR4354. (D) Extent of WNV fusion with increasing concentrations of antibody. The extent of fusion was determined at 60 s upon acidification and is shown as a percentage of the control (no antibody) (pH 6.3). White bars, isotype-matched control MAb; dark gray bars, CR4348; light gray bars, CR4354. Representative fusion data for at least three independent experiments are shown.

or after virus binding to a monolayer of Vero cells, and infection was measured by a plaque reduction assay. As expected, CR4348 and CR4354 efficiently neutralized infection when premixed with virus (Fig. 4A). Both MAbs also inhibited WNV infection when added after virus adsorption to the cell surface, indicating that at least part of their neutralizing activity was at a postattachment step of the viral life cycle. When added after attachment, CR4354 was more potent than CR4348 in neutralizing infection. Importantly, no pre-or postattachment neutralization was detected with a nonbinding isotype control, and a largely postattachment pattern of inhibition was seen with Hu-E16 (Fig. 4A), as observed previously (57).

pH sensitivity of CR4348 and CR4354 binding.

The exposure of flavivirus virions or SVP to acidic pH in solution or in the endosome prompts a rapid structural rearrangement (7, 19, 49, 77). Given that CR4348 and CR4354 preferentially recognize epitopes present on virions and SVP but not on recombinant E proteins (Fig. 2), we hypothesized that changes in the oligomeric arrangement of E that are associated with shifts in pH might alter immunoreactivity. To test this, we used our SVP capture ELISA, exposing particles to different pH (pH 6.0 or 7.4) conditions for 30 min prior to pH normalization (pH 7.4) and incubation with MAbs. Notably, our control neutralizing MAb, Hu-E16, did not show pH-sensitive binding (Fig. 4B). In contrast, the exposure of SVP to pH 6.0, which should promote E protein rearrangement and irreversible trimer formation, significantly reduced the levels of binding of both CR4348 and CR4354 ($P \leq 0.003$).

CR4348 and CR4354 inhibit WNV fusion. Since both CR4348 and CR4354 have the capacity to neutralize WNV infection at a postattachment step, we investigated whether these MAbs would also affect WNV fusion. To evaluate this, we utilized a model liposome fusion assay with pyrene-labeled virus that was previously used to monitor the fusion dynamics of TBEV (9, 19, 75–77). Briefly, pyrene-labeled WNV was preincubated with different concentrations of MAbs for 1 h at RT and then mixed with liposomes at 37°C. Fusion was triggered by the acidification of the mixture to pH 6.3, which is the optimal pH for WNV fusion (B. Moesker, J. Wilschut, and J. Smit, unpublished observations). In the absence of antibodies or in the presence of 50 nM (7.5 μg/ml) of a nonbinding control MAb, fusion was essentially complete within seconds upon acidification. In contrast, preincubation of the virus with CR4348 or CR4354 potently inhibited fusion activity (Fig. 4C). Both MAbs reduced the extent of fusion in a dose-dependent manner (Fig. 4D). CR4354 inhibited fusion at concentrations of 1 nM (0.15 μg/ml) and above, whereas for CR4348, a minimal concentration of 10 nM (1.5 μg/ml) was required. This difference in potency was also reflected in the maximum level of fusion inhibition at saturating antibody concentrations. CR4354 and CR4348 neutralized ~85% and 65% of the virus particles, respectively (Fig. 4D). Thus, in the model liposome assay, CR4348 and CR4354 can block the low-pH-catalyzed fusion of the majority of WNV virions; this supports the idea that these MAbs act at a postattachment step. Nonetheless, even under conditions of saturating antibody concentrations, a fraction of the virus particles resists complete inhibition by these MAbs, although the rate of fusion of the residual fractions is substantially lower than that in the absence of antibody.

Generation of neutralization escape mutants.
Because CR4348 and CR4354 recognized a determinant on the WNV virion or SVP, which is not readily apparent on recombinant or yeast-displayed forms of E, we generated neutralization escape mutants to further define their epitopes. After three sequential virus passages on Vero cells under CR4348 or CR4354 selection (25 μg/ml), WNV was no longer neutralized by these MAbs in plaque reduction assays (Fig. 5A). To determine the mutations that conferred the escape phenotype, RNA sequences obtained from plaque-purified escape variants were compared to the wild-type WNV sequence derived from virus passaged in parallel in the absence of antibody selection. All (five of five) sequences from CR4348 escape variants contained the same single-nucleotide change encoding a T208I mutation in the E protein; in addition, two of the five sequences also had an independent H246Y mutation in the E protein. In contrast, all (15 of 15) CR4354 escape variants contained a single-nucleotide mutation, encoding a K136E mutation in the E protein; no other nucleotide changes were observed in any of the CR4354 variants.

To establish that these amino acid substitutions conferred the neutralization escape phenotypes observed, we utilized two reverse-genetic systems. First, RVP with single amino acid mutations were generated and analyzed for MAb neutralization. Whereas Hu-E16 neutralized all mutant and wild-type RVP equivalently, the T208I and K136E RVP were not efficiently neutralized by CR4348 and CR4354, respectively, even at concentrations of 15 μg/ml of MAb (Fig. 5B). H246Y RVP were neutralized by CR4348 but only at the highest doses of antibody tested; the concentration at which 50% inhibition (EC_{50}) occurred was increased by >31-fold ($P < 0.02$) compared to that of wild-type RVP (Fig. 1B).

As an independent confirmation, we introduced these substitutions into a wild-type, infectious New York 1999 WNV cDNA clone (NY99ic) (5) to generate mutant viruses. Analogous results were obtained with all genetically engineered mutant WNV and MAbs using a plaque reduction assay on BHK21-15 cells (Fig. 5C). Thus, results of experiments with RVP and infectious cDNA clones confirmed the sequencing results and suggest that CR4348 and CR4354 likely bind distinct epitopes on the WNV E protein. To directly determine the contributions of these amino acids to MAb binding, we used mutated NY99ic WNV in a capture ELISA (Fig. 5D). As expected, Hu-E16 recognized all three variant viruses. CR4354 failed to bind to WNV with a K136E mutation but did recognize the other viruses, establishing that a change in K136 confers a loss-of-binding phenotype. In contrast, no decrease in the level of CR4348 binding was observed for the single T208I or H246Y variant or a double T208I H246Y variant (data not shown). Thus, these two individual mutations, which strongly impact CR4348 neutralization, do not alter MAb binding in the capture ELISA.

To gain a better understanding of why CR4348 and CR4354 recognized intact virions or subviral particles but not recombinant E proteins, we mapped the residues that conferred escape from MAb neutralization onto the existing WNV E protein crystal structures (30, 58). K136, T208, and H246 are all solvent-accessible residues (Fig. 6A). K136 is located at the end of DI adjacent to the hinge between DI and DII. This hinge rotates 20° to 30° during structural rearrangements of the E protein associated with low-pH-induced viral fusion in late endosomes (7, 49). The flexibility of the DI-DII hinge may be less dynamic on an icosahedral virion or SVP than the soluble recombinant protein, possibly explaining the differential reactivity of the

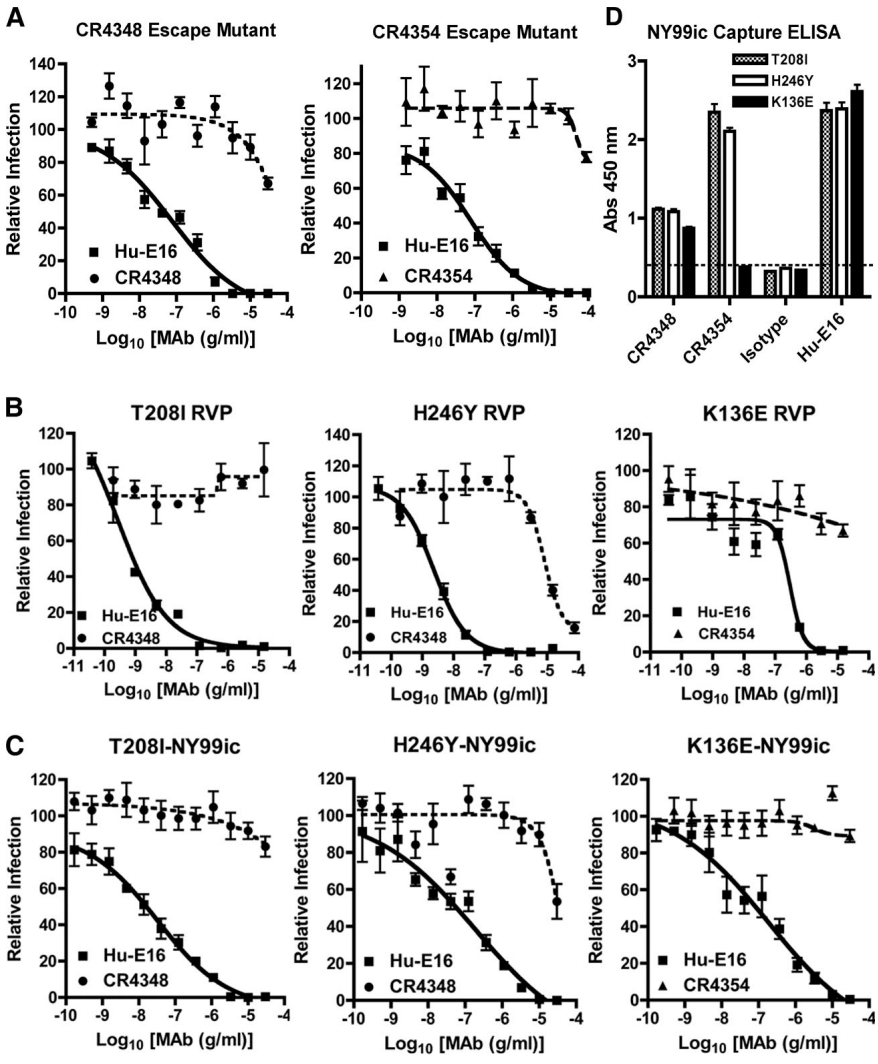


Figure 5. Characterization of neutralization escape mutants. (A) PRNT performed in duplicate with virus after three passages under selection of either CR4348 (left) or CR4354 (right) on Vero cells. A reduced neutralization capacity was observed compared to that of Hu-E16. (B and C) Confirmation of a resistant phenotype with reverse-genetic RVP or infectious cDNA clone assays. Mutated RVP (B) and infectious WNV (C) were used to confirm that single amino acid substitutions of T208I (left), H246Y (middle), and K136E (right) in the E protein could reduce or eliminate neutralization by the selecting MAb. In each series of experiments, the reduced neutralizing capacity of the indicated human MAb was compared to the neutralizing capacity of Hu-E16, which maps to a distinct epitope on the DIII-lr. The data are combined data from two or three separate experiments performed in duplicate or triplicate. Error bars indicate standard deviations. Lines represent curve fits generated by nonlinear regression analysis. (D) A capture ELISA was used to detect the binding of MAbs to mutated (K136E, T208I, and H246Y) WNV virions. Microtiter plates were coated with murine DIII-lr MAb, incubated with mutated virus, and detected with the indicated human IgG1 MAbs. Dashed lines indicate the background of the assay with an isotype control human MAb. The data are representative of data from three separate experiments performed in triplicate. Error bars indicate standard deviations. Abs, absorbance.

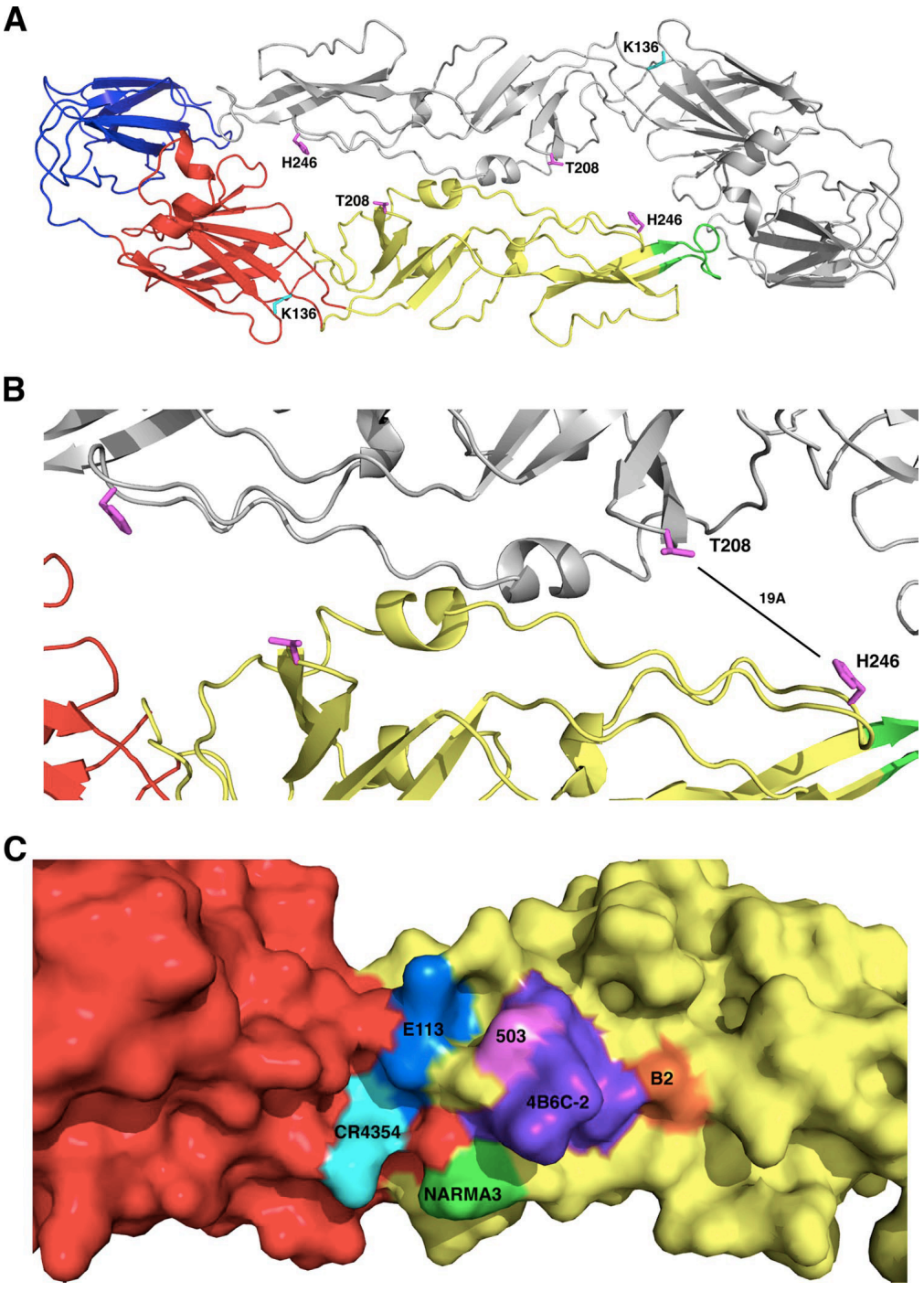


Figure 6. Structural mapping of MAb epitopes. (A) WNV E protein dimer, with K136 (CR4354) in blue and T208 and H246 (CR4348) in magenta. (B) Close-up view of the WNV E DII dimer interface, with the CR4348 epitope highlighted in magenta. (C) Surface display model of the WNV E DI-DII hinge region with epitope recognition sites of CR4354 and the corresponding WNV residues of other antiflavivirus MAbs (E113, 503, NARMA3, B2, and 4B6C-2) listed in Table 4. Note that residue 136 is labeled CR4354 but is also part of the 503 epitope, and residue 126 is labeled B2 but is also part of the 503 and 4B6C-2 epitopes.

CR4354 epitope. In contrast, the conformationally sensitive nature of CR4348 binding may be due to a preferential reactivity with WNV E protein dimers. T208 and H246 are present in DII and, although relatively distant (43 Å) within an individual E protein monomer, are separated by 19 Å across the dimer interface, which is within the spatial limits of an antibody footprint (Fig. 6B). It is important that in contrast to the TBEV and DENV E proteins, which readily form dimers in solution, the soluble WNV E protein is largely monomeric (30, 58). Consistent with the mapping and binding data that suggest that the two human MAbs recognize distinct epitopes, CR4348 efficiently neutralized the K136E WNV variant, and CR4354 inhibited infection of the T208I and H246P variants (data not shown).

Given these mapping data, we hypothesized that CR4348 might recognize oligomeric forms of the E protein that are not prevalent in our purified recombinant preparation. As mentioned above, ELISA plates coated with the WNV E protein overnight at 4°C showed no immunoreactivity with either CR4348 or CR4354 (Fig. 2B). However, coating plates with the E protein at 37°C resulted in a modest yet significant signal by CR4348, possibly due to oligomeric interactions between E proteins that occur more favorably at a higher temperature, prior to adsorption (Fig. 2B).

Phenotype of WNV variants in cell culture.

Comparisons of E protein amino acid sequences of WNV isolates that varied geographically and temporally showed that T208 and H246 were completely conserved. K136 was completely conserved among lineage 1 WNV isolates but varied in the less-virulent lineage 2 and 3 strains, with alanine and serine substitutions, respectively (data not shown). To evaluate whether the mutations associated with the neutralization escape of CR4348 and CR4354 were functionally important for virus replication and affected viral virulence, growth analyses using BHK21-15 cells was performed. The plaque morphologies of the three mutant viruses (K136E, T208I, and H246Y) in BHK21-15 cells was compared to that of wild-type WNV strain New York 1999. The wild type and K136E and T208I variants had similar large plaque morphologies, whereas the H246Y mutant had a small plaque phenotype (data not shown).

In vivo protection studies.

Passive transfer of neutralizing MAbs against WNV confers protection against disease in mice (22, 59, 61, 69, 78) and hamsters (53, 54). To evaluate the potency of human MAbs, protection studies were performed using wild-type C57BL/6 mice after infection with WNVNY99ic. Analogous to results with Hu-E16 (59), prophylaxis with low (1.4 and 0.4 μg, respectively) doses of CR4348 and CR4354 completely protected mice against lethal WNV encephalitis (Table 1). No protective effect was observed in these or other previously reported studies (17, 59, 78) using isotype control antibodies. To characterize the protective activity of the more potent of the two neutralizing MAbs, CR4354, in greater detail, infection studies with wild-type and K136E escape variant WNV were repeated using highly susceptible 3- to 4-week-old NIH Swiss mice (Table 2); this strain was selected because WNV morbidity and mortality show a linear virus dose dependence (4). Consistent with the cell culture plaque morphology data, the lethalities of wild-type and K136E WNV-NY99ic were not different, with 50% lethal dose

Table 1. Effect of MAb pretreatment on survival of C57BL/6 mice[b]

MAb	Dose (μg)	No. of survivors/ total no. of mice	% Survival	*P* value[a]
PBS		4/20	20	
CR4348	14	4/4	100	≤0.01
CR4348	4.2	5/5	100	≤0.01
CR4348	1.4	5/5	100	≤0.01
CR4348	0.42	3/5	60	0.13
CR4348	0.14	3/5	60	0.10
CR4348	0.042	2/5	40	0.31
CR4348	0.014	1/5	20	0.70
CR4354	14	5/5	100	≤0.01
CR4354	4.2	5/5	100	≤0.01
CR4354	1.4	5/5	100	≤0.01
CR4354	0.42	5/5	100	≤0.01
CR4354	0.14	4/5	80	0.02
CR4354	0.042	2/5	40	0.21
CR4354	0.014	1/5	20	0.57

[a] *P* values were determined using the log-rank test and are compared to the PBS controls.
[b] C57BL/6 mice were pretreated with the indicated dose of human MAb at day -1 by intraperitoneal injection. On day 0, mice were infected with WNV by the subcutaneous route and monitored for survival.

values of 0.28 and 0.24 PFU, respectively (P > 0.8). Sequencing of viral RNA from brains of mice infected with wild-type or K136E variant WNV-NY99ic revealed no amino acid changes: WNV in the brain after infection with wild-type and mutant viruses contained K and E residues at position 136, respectively (data not shown). To confirm the neutralization escape phenotype, mice were pretreated with 50 μg (100 times the minimum protective dose in C57BL/6 mice) of CR4354 MAb 1 day prior to infection with ~3x10[1] PFU of wild-type or K136E variant WNV-NY99ic. CR4354 protected 94% (15 of 16) of mice from wild-type NY99ic challenge, whereas only 19% (3 of 16) of mice challenged with K136E-NY99ic survived despite CR4354 prophylaxis. In contrast, Hu-E16 protected nearly all mice after infection with either the wild type or K136E-NY99ic. Collectively, these data confirm the highly protective activity of CR4354 in vivo and the CR4354 escape phenotype of the K136E mutation.

DISCUSSION

Previous studies suggested that antibodies that map to the DIII-lr epitope do not account for the majority of neutralizing activity in serum from human patients or horses (60, 70). In this study, we characterized the functional properties of two neutralizing human MAbs, CR4348 and CR4354, that recognize distinct epitopes. Both MAbs inhibit WNV infection in vitro and in vivo but did not recognize closely or distantly related flaviviruses, including St. Louis encephalitis virus or DENV (M. Throsby, M. Vogt, and M. Diamond, unpublished results). Biochemical studies demonstrate that these MAbs have similar but not identical profiles: they bind to

Table 2. Effect of the K136E substitution on MAb protection of NIH Swiss mice[b]

MAb	NY99ic WNV	No. of survivors/ total no. of mice	% Survival	*P* value[a]
PBS	Wildtype	4/16	25	
Hu-E16	Wildtype	16/16	100	<0.0001
CR4354	Wildtype	15/16	94	<0.0001
PBS	K136E	1/16	6	
Hu-E16	K136E	15/16	94	<0.0001
CR4354	K136E	3/16	19	0.37

[a] *P* values were determined using the log-rank test and compared to the PBS controls for each virus.
[b] NIH Swiss mice were pretreated with 50 μg of human MAb (Hu-E16 or CR4354) at day -1. On day 0, mice were infected with 3 x 10^1 PFU/mouse of wild-type NY99ic or K136E-NY99ic virus and monitored for survival.

conformationally sensitive epitopes on E proteins displayed on virions or SVP in a pH-sensitive manner but recognize recombinant E proteins in solution or displayed on the surface of yeast poorly, if at all. Functional experiments suggest that both human MAbs neutralize infection primarily at a postattachment stage in the viral life cycle, specifically through the inhibition of viral fusion with the endosomal membrane.

The CR4354 recognition site was established by neutralization escape and reverse-genetic experiments and localized to the DI-DII hinge interface at residue K136. This hinge is highly flexible, as the angle between DI and DII on DENV E rotates 27° during the transition from the immature to the mature state after the furin-mediated cleavage of prM (87). The hinge then rotates back 30° during the prefusion-to-postfusion domain rearrangement (7, 49). Two X-ray crystal structures of similarly prepared WNV E proteins also have distinct DI-DII hinge angles, differing by 5° (30, 58). This inherent flexibility of the DI-DII hinge could explain the lack of CR4354 binding to soluble and yeast surface-displayed E, as these recombinant E proteins may not display the native hinge that is found on virions or SVP. Indeed, the exposure of SVP to acidic conditions that alter the DI-DII hinge angle significantly reduced the level of binding of CR4354.

Although the characteristics of CR4354 appear relatively unique among anti-WNV MAbs, several other antiflavivirus neutralizing MAbs that localize to this region have been described (Table 3). The anti-WNV MAb that maps closest to this region is mouse MAb E113, which binds a determinant along the DI-DII hinge interface at residues E49 and K208 (Fig. 6C). E113 is protective in vitro (EC_{50} of 0.25 μg/ml) and in vivo, but unlike CR4354, E113 binds recombinant and yeast-displayed E proteins efficiently (61). The importance of this epitope for antibody neutralization is reflected by the characterization of several DI-DII-inhibitory antiflavivirus MAbs. Notably, MAbs (503, NARMA3, and B2) that recognize the DI-DII hinge are strongly inhibitory against the closely related JEV, which is a member of the same serogroup as WNV (21, 27, 35, 52).

Neutralization escape experiments suggested that T208 and H246 are recognition sites near the DII dimer interface for MAb CR4348. The importance of these residues for neutralization was confirmed using reverse-genetic approaches by introducing these mutations into WNV

Table 3. Flavivirus MAbs that localize to the DI-DII hinge interface

MAb	Virus	Epitope(s)	Immunogen(s)	Source of MAb	Neutralization[a]		Reference(s)
					In vitro	In vivo	
CR4354	WNV	K136	Natural infection	Human	++	++	78, this paper
E113	WNV	E49, K280	Infectious virus + recombinant E-protein	Mouse	+	+	61
503	JEV	S275, K136, I126	Infectious virus	Mouse	++	++	34, 35, 52
NARMA3	JEV	Q52	Infectious virus	Mouse	++	ND	27, 37
B2	JEV	I126	Vaccine + infectious virus	Chimpanzee	++	++	21
4B6C-2	Murray Valley encephalitis virus	A126, R128, F274, S276, S277	Purified virus	Mouse	++	++	28, 47

[a] ++, greater than 90% inhibition via any in vitro assay or capable of 90% protection in any lethal in vivo model; +, any significant in vitro neutralization or in vivo protective capacity; ND, not determined.

RVP and the infectious cDNA clone. Although the mutation of these two residues abolished neutralization, they did not prevent MAb CR4348 binding in capture ELISAs, suggesting that other amino acids that were not revealed in our selection experiments contribute to the epitope. While T208 and H246 lie spatially far apart (~43 Å) within the E monomer, likely beyond the footprint of the antibody paratope, they reside significantly closer (~19 Å) across the dimer interface. Thus, CR4348 may recognize an epitope that is sensitive to the oligomeric state of the E protein. Consistent with this, CR4348 does not stably bind WNV E on yeast cells or in the solid phase when adsorbed at a low temperature (4°C). However, when WNV E was adsorbed onto microtiter plates at 37°C, we reproducibly observed modest levels of binding. Furthermore, CR4348 binds poorly to SVP that have been exposed to mildly acidic solutions; a decrease in pH induces a structural rearrangement in flavivirus E proteins, resulting in dimer dissociation and trimer formation (7, 49).

The CR4348 MAb epitope is structurally and functionally unique among characterized anti-WNV MAbs. Although CR4348 recognizes residues at the DII dimer interface that are proximal to mouse anti-WNV E MAb E100 (61), the two differ in several respects: E100 maps more distal from DI at residue H263, only modestly neutralizes WNV in cell culture (EC_{50} 10 μg/ml), recognizes both soluble and yeast forms of E, and shows a quite limited protective efficacy against lethal WNV infection in mice. However, a DII dimer interface MAb with more-similar properties for the distantly related flavivirus TBEV has been described (Table 4). MAb A5 (26) maps to residue E207 along the dimer interface (45), is strongly neutralizing in culture (26), and partially blocks TBEV fusion in a pyrene excimer liposome fusion assay (74). Moreover, the binding of at least some cross-reactive neutralizing flavivirus MAbs (e.g., 4G2 and 6B6C-1) that map to the fusion peptide in DII are also affected by mutations of residues (E231) along a dimer interface (10). One speculation as to why so few MAbs with these functional properties have been described is that they may not be identified with screens or immunization protocols using recombinant E proteins.

Table 4. Flavivirus MAbs that localize to the DII dimer interface

MAb	Virus	Epitope(s)	Immunogen(s)	Source of MAb	Neutralization[a]		Reference(s)
					In vitro	In vivo	
CR4354	WNV	T208, H246	Natural infection	Human	++	++	78, this study
E100	WNV	H263	Infectious virus + purified E	Mouse	+	+	61
A5	TBEV	E207	Solubilized virus	Mouse	+	ND	26, 45

[a] ++, greater than 90% inhibition via any in vitro assay or capable of 90% protection in any lethal in vivo model; +, any significant in vitro neutralization or in vivo protective capacity; ND, not determined.

One limitation to mapping analyses by neutralization escape, which may be important for MAbs that show a loss of function with mutations in a flexible hinge, is the inability to exclude a distal binding site that is modulated allosterically by the hinge. A lack of binding to yeast or soluble E could reflect that these recombinant forms of E are truncated at amino acid 415 and lack the C-terminal stem-anchor and transmembrane regions. The lack of an identification of an escape mutant in the highly conserved C-terminal regions could be due to a poor viability of these variants. Unfortunately, because these MAbs bind poorly to recombinant E proteins, cocrystallography (44, 57), nuclear magnetic resonance (82), or saturation mutagenesis (23, 24, 46) approaches to identify the structural epitope are not possible. Instead, cryo-electron microscopy studies with Fab-virion complexes (31, 44) are planned to confirm the location of the epitope on the virion. Such experiments will be especially important for MAbs like CR4348, which shows a loss of neutralization but not binding with mutations at the DII dimer interface.

We previously described E16, a strongly neutralizing WNV specific mouse MAb that maps to the DIII-lr of the E protein (59) and inhibits infection by blocking viral membrane fusion in endosomes (B. Thompson, B. Moesker, J. Wilschut, J. Smit, M. Diamond, and D. Fremont, unpublished data). Although CR4348 and CR4354 also strongly neutralize infection and affect a postattachment step in the viral life cycle, they likely inhibit WNV by a mechanism distinct from that of E16. CR4354 appeared to inhibit infection almost equivalently when it was added before or after attachment. In contrast, CR4348 and E16 showed somewhat enhanced neutralizing activity when added prior to attachment. Moreover, in the liposomal model system, E16 completely blocked fusion at different pH values ranging from pH 6.3 to 5.0, consistent with ELISA and surface plasmon resonance data showing pH-independent binding of E16 to SVP (Thompson et al., unpublished). In contrast, CR4354 and CR4348 do not completely block low-pH-induced fusion of the virus with liposomes, with some residual fusion activity observed. This residual fusogenic activity in the setting of saturating concentrations of CR4354 and CR4348 may be due to heterogeneity among the WNV virions. Indeed, we also observed a small resistant fraction of virions in classical $PRNT_{50}$ analyses under conditions of antibody saturation, with ~10% and 20% of the virus remaining infectious in the presence of high concentrations of CR4354 and CR4348, respectively (Fig. 1A). At present, the molecular basis for the differences in residual infectivity or fusogenic activity of the two experimental systems under conditions of an excess of CR4354 and CR4348 remains uncertain. The residual non-neutralized fractions, however, are not likely related to the maturation stage of the virus (56), since mature and partially immature WNV RVP are neutralized equivalently by these MAbs.

More-detailed analyses of antibody-virion structures and the precise pH dependency of the inhibition are planned to further define the mechanisms of neutralization.

For WNV and other flaviviruses, passive immunotherapy has been shown to protect small animals against lethal infection even when administered several days after infection (22, 59, 68). As recent studies suggested that resistance to monotherapy with MAb E16 can occur in vivo (84), the use of combinations of neutralizing MAbs that recognize distinct epitopes may be advantageous, as was demonstrated previously for a therapeutic antibody cocktail against rabies virus (2). The characterization of potently neutralizing MAbs like CR4348 and CR4354 that map to distinct regions and inhibit virus by different mechanisms suggests that this may be feasible.

ACKNOWLEDGMENTS

We thank members of our laboratories for helpful discussions, James Brien for experimental assistance, Jolanda Smit for critical comments and suggestions, and Syd Johnson for preparation of MAb Hu-E16.

This work was supported by grants from the NIH (grant U01 AI061373 [M.S.D.]) and the Midwest Regional Center of Excellence for Biodefense and Emerging Infectious Diseases Research (grant U54 AI057160).

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prM-antibody renders immature West Nile virus infectious in vivo

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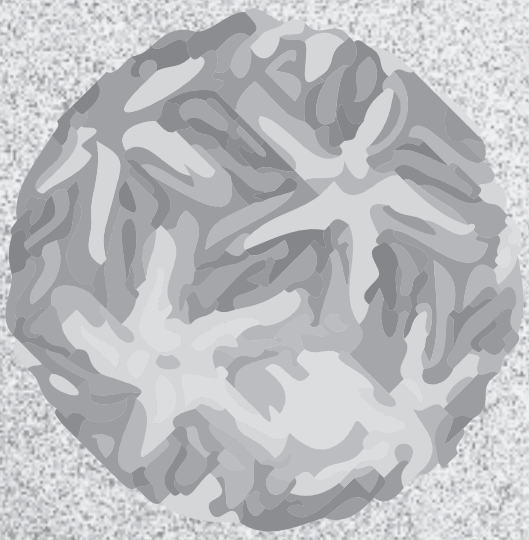
Journal of General Virology (2011). *In press*



Chapter 6

SUMMARY

West Nile virus (WNV) is a member of the Flaviviridae and a neurotropic pathogen responsible for severe human disease. Flavivirus infected cells release virus particles that contain variable numbers of precursor membrane (prM) protein molecules at the viral surface. Consequently, antibodies are produced against the prM protein. These antibodies have been shown to activate the infectious potential of fully immature flavivirus particles in vitro. Here, we provide in vivo proof that prM antibodies render immature WNV infectious. Infection with antibody-opsonized immature WNV particles caused disease and death of mice and infectious WNV was found in the brains and sera.

prM-opsonized immature West Nile virus is infectious in mice

West Nile virus (WNV) is a mosquito-borne flavivirus and an emerging pathogen responsible for encephalitis and neurologic disease in humans (Mackenzie *et al.*, 2004). Flaviviruses, which also include dengue virus (DENV) and tick-borne encephalitis (TBEV) virus, are enveloped, single-stranded RNA viruses and contain 3 structural proteins, capsid (C), envelope (E) and membrane (M). In mature virions, E is organized as 90 homodimers that lie flat against the viral surface forming a "smooth" protein shell (Kuhn *et al.*, 2002).

Flaviviruses infect cells via receptor-mediated endocytosis, which is mediated by the E glycoprotein (Lindenbach, 2001; Mukhopadhyay *et al.*, 2005; van der Schaar *et al.*, 2007). Following RNA replication, immature virions are formed which contain a precursor membrane protein (prM) in a heterodimeric configuration with E that extend as 60 trimeric spikes from the viral surface (Kuhn *et al.*, 2002). These newly formed particles mature during virus egress through the secretory pathway, where cleavage of prM by a furin-like protease generates infectious particles (Stadler *et al.*, 1997). This cleavage is known to be fairly inefficient as the prM content of virus particles released from DENV and WNV-infected cells is approximately 30% (Moesker *et al.*, 2010; Zybert *et al.*, 2008). Furthermore, the prM content is variable on a per particle basis as recent studies showed that both fully immature as well as partially immature, or nearly mature, particles exist in wild-type (wt) preparations of DENV and WNV (Junjhon *et al.*, 2010; Li *et al.*, 2008; Stadler *et al.*, 1997).

Recent studies showed that anti-prM is a major component of the serological response in DENV infection (Cardosa *et al.*, 2002; Dejnirattisai *et al.*, 2010). The role of prM antibodies during flavivirus infection was long not understood as numerous functional studies revealed that immature prM-containing flavivirus particles are noninfectious (Elshuber & Mandl, 2005; Stadler *et al.*, 1997). Indeed, studies on TBEV, DENV and WNV have shown that cleavage of prM to M is required to activate the membrane fusion machinery of the virus (Guirakhoo *et al.*, 1991; Moesker *et al.*, 2010; Stadler *et al.*, 1997; Zybert *et al.*, 2008). Interestingly, however, we recently observed that prM antibodies bind to fully immature DENV particles and facilitate cellular entry through the Fc-receptor in a furin-dependent manner (Rodenhuis-Zybert *et al.*, 2010). Furthermore, antibodies against prM have been shown to enhance wt DENV infection (Huang *et al.*, 2006) and the levels of prM antibodies were found to be higher in patients with secondary infection compared to sera from primary DENV infection (Lai *et al.*, 2008). Moreover, a recent study showed that human anti-prM antibodies fail to efficiently neutralize immature DENV infection in primary monocytes and enhance infection 20 to 70% even at high concentrations (Dejnirattisai *et al.*, 2010). Also, human anti-prM antibodies did not significantly protect AG129 interferon-deficient mice from WNV infection (Calvert *et al.*, 2011). This indicates that during natural flavivirus infection, depending on the proportion of mature and immature virions present in a population, prM antibodies may set the stage to antibody-dependent enhancement of infection. This also suggests that immature virus could potentially be an important component of flavivirus infection and as such contribute to the development of disease.

In this study, we investigated the ability of prM antibodies to facilitate immature flavivirus infection in vivo using the well-established model of West Nile virus (NY99) infection in mice. To this end, wt WNV was generated in baby hamster kidney (BHK) cells and immature WNV

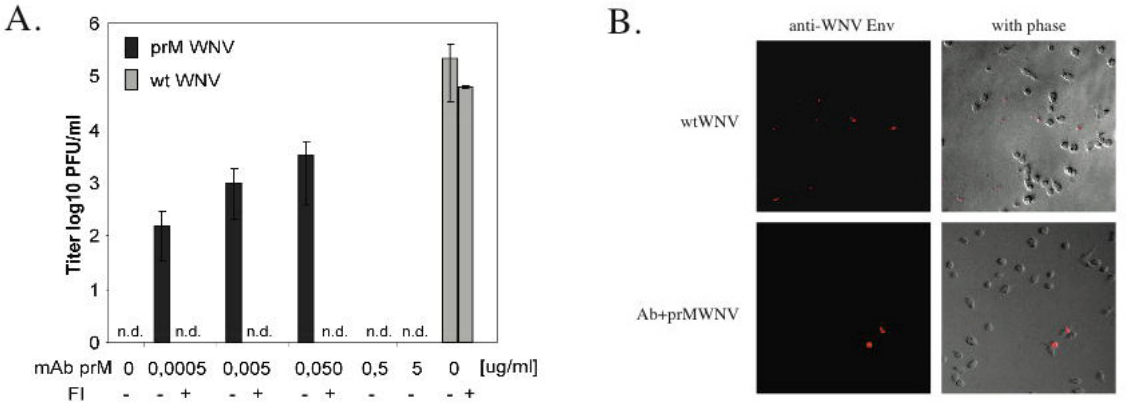


Figure 1. prM-specific antibody Ab25888 renders immature WNV particles infectious in a furin-dependent manner. A. P388D1 cells were infected with immature WNV at MOG 10 in the presence of increasing concentrations of anti-prM Ab25888. When indicated, 25 μM furin inhibitor was added to the cells prior and during infection. At 26 hpi the supernatant was harvested and the production of virus particles was measured by plaque assay on BHK-15 cells. Data is expressed as means of duplicate experiments. The error bars respresent standard deviations; (n.d.) denotes "not detectable". B. WNV-infected P388D1 cells were fixed with 4% paraformaldehyde and stained with an antibody against WNV envelope protein conjugated to TRITC. Representative images are shown for WT WNV infection (top panels) and prM antibody-opsonized immature WNV (bottom panels).

(prMWNV) was generated in furin-deficient LoVo cells, as described before (Moesker *et al.*, 2010). We previously reported that LoVo-derived WNV is almost completely immature, the prM content being 87 ± 7% (Moesker *et al.*, 2010). The specific infectivity of LoVo-derived WNV was determined by measuring the number of infectious units by plaque assay on baby hamster kidney (clone 15) cells (BHK-15) and the number of genome-containing particles (GCPs) by quantitative PCR (qPCR). To determine the number of GCPs, viral RNA was extracted by use of a QIAamp viral RNA mini kit (QIAGEN, Venlo, The Netherlands). Next, cDNA was synthesized with RT-PCR and the qPCR reaction was performed similar as described before for DENV (van der Schaar *et al.*, 2008). For WNV, the forward primer 5'-GTT GGC GGC TGT TTT CTT TC-3', and the reverse primer 5'-GGG ATC TCC CAG AGC AGA ATT-3' and a TaqMan probe 5'-FAM-AAT GGC TTA TCA CGA TGC CCG CC-TAMRA-3' (Eurogentec, Seraing, Belgium) was used. The concentration of GCPs was determined by use of a standard curve based on a cDNA plasmid encoding the nonstructural genes of WNV NY99 (kind gift from Dr. G.P. Pijlman, Wageningen University, The Netherlands). The p.f.u.-to-particle ratio of wt WNV was on average 1:330 (n=2), which is in agreement with earlier reports (Moesker *et al.*, 2010; Wengler & Wengler, 1989). LoVo-derived virus was found to be virtually non-infectious, the p.f.u.-to particle ratio being approximately 30,000-fold lower compared to that of wt WNV.

Next, the infectious properties of prMWNV particles in presence of increasing concentrations of prM-specific antibody Ab25888 (Abcam, MA) were determined in Fc-receptor expressing P388D1 mouse macrophage-like cells (Figure 1). Virus or preformed virus-antibody complexes were added to P338D1 cells at a multiplicity of genome-containing particles (MOG) of 10. At 24 hours post-infection (hpi), the media was harvested and the number of produced

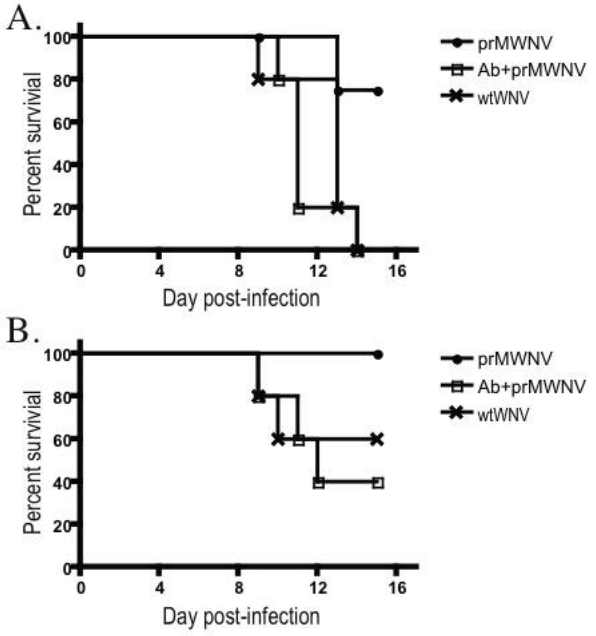


Figure 2. prM-specific antibody Ab25888 renders prMWNV particles infectious in vivo. Mice were infected with wt WNV, prMWNV or prM antibody-opsonized (Ab) prMWNV via IP injection. Curves show percent survival up to 16 days post-infection. For each curve, n=5 and P < 0.05. Top panel is 3.4 x 10^6 GCPs WNV per mouse, bottom panel is 3.4 x 10^5 GCPs WNV per mouse.

viral particles was measured by plaque assay on BHK-15 cells. Figure 1 shows that prMWNV is non-infectious in P388D1 cells, as no plaque forming units are detected in the absence of antibodies. Interestingly, and consistent with DENV (Rodenhuis-Zybert *et al.*, 2010, Dejnirattisai *et al.*, 2010), prM antibodies render fully immature WNV particles infectious (Figure 1A). The infectivity is significantly increased at an antibody concentration of 0.0005, 0.005, 0.05 µg/ml. At high antibody concentrations, no enhancement of infection is observed. Subsequently, immunofluorescence studies were performed to quantify the number of infected cells for wt and prM-opsonized immature virus (Figure 1B). To this end, cells were fixed at 24 hpi with 4% paraformaldehyde and stained with a WNV E antibody conjugated with TRITC (L2, CT). The percentage of infected cells was determined by counting the number of infected fluorescently labeled cells per a total of 1,000 cells in 3 separate experiments. We found that 4.77% +/- 1.02 % of the cells are infected with prM-opsonized immature WNV and 7.23% +/- 1.5% with wt under the conditions of the experiment. This indicates that the specific infectivity of antibody-opsonized immature WNV at conditions of efficient antibody-dependent enhancement of infection is just over half of that of wt WNV. Enhancement of infection of prMWNV particles opsonized with antibodies was seen in multiple Fc receptor expressing cell lines including human erythroleukemic K562 cells and human leukemic monocyte lymphoma U937 cells (data not shown). To determine if the observed enhancement is dependent on the enzymatic activity of furin, P388D1 cells were treated with 25 µM of the furin-specific inhibitor decanoyl-L-arginyl-L-valyl-L-lysyl-L-arginyl-chloromethylketone (decRRVKR-CMK, Calbiochem, Darmstadt, Germany) prior to and during infection, as described before (Rodenhuis-Zybert *et al.*, 2010). Consistent with our results on DENV, enhancement of infection by prM antibody coated prMWNV particles is strictly dependent on furin activity (Figure 1A).

After confirming the infectivity of antibody-opsonized prMWNV in vitro, we wanted to determine if the virus was infectious in vivo. If antibody-opsonized prMWNV particles are infectious in vivo, wt WNV particles should be produced after a single round of infection leading to encephalitis and death in mice (Bai *et al.*, 2009; Wang *et al.*, 2004). Furthermore, if antibody-opsonized prMWNV can cause similar mortality due to encephalitis, it may indicate

that these virions are able to contribute to disease and pathogenesis during natural infection. To test this, prMWNV was incubated with PBS alone or 10 µg/mL prM antibody (Ab) for one hour at 37°C. Infection was done via IP injection into strain c57L/b6 mice. The final concentration of opsonized virus was 3.4 x 10^5 or 3.4 x 10^6 GCPs per mouse. The same number of GCPs of wt WNV was used to infect a control group of mice. The number of GCPs given to the mice is calculated on the basis of 10^3 or 10^4 infectious particles for wt WNV, respectively. For each infectious condition *n*=5. The mice injected with either 3.4 x 10^5 or 3.4 x 10^6 GCPs of prMWNV alone did not show any signs of infection and had a much greater survival rate than mice infected with wt WNV. Interestingly, the prM Ab-opsonized prMWNV caused similar disease and death as with the wt WNV infected-mice, demonstrating that prM antibodies render prMWNV particles infectious (Figure 2).

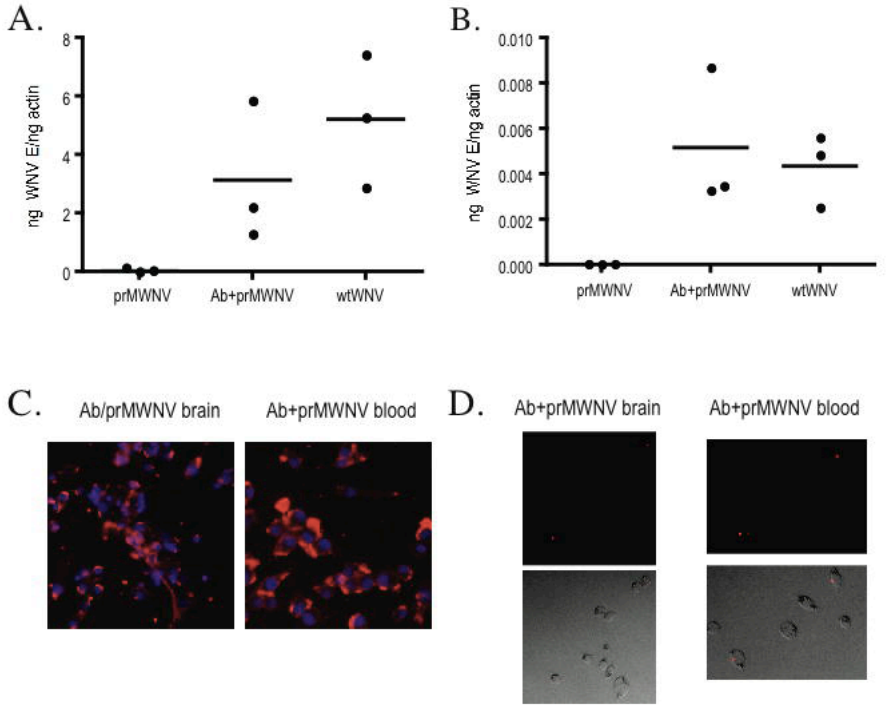


Figure 3. Brain homogenates and blood from prM-opsonized prMWNV are infectious and infected cells produce new infectious WNV. Mice were infected with 10^7 GCPs of either wt WNV, prMWNV, or prM antibody-opsonized (Ab) prMWNV via IP injection. A & B. RNA was isolated from brain (A) or blood (B) of infected mice and qPCR was used to detect the WNV envelope (E) gene. Data shown as ng WNV E/ng beta-actin, *n*=3 per group. C & D. Brains from mice infected with prM-specific antibody-opsonized WNV were homogenized in 1mL PBS(-) and used to infect P388D1 cells (C, left panel). Blood from the same mice were used to infect P388D1 cells (C, right panel). 7 days post-infection, cell-culture supernatants were used to infect new P388D1 cells (D). Cells were fixed with 4% paraformaldehyde and stained with an antibody against WNV E conjugated to TRITC (red). A DAPI counterstain was used to show the nucleus (C). Representative images are shown for each infection.

To confirm that the mortality in the mice was due to WNV infection in the brain, an additional group of mice were infected with either wt WNV, prMWNV alone or Ab-opsonized prMWNV. Blood was drawn from the mice on day 3 post-infection and on day 7 post-infection, the brains were collected and homogenized in 1 mL PBS (-). RNA was isolated from both whole blood and brain homogenate and the cDNA made was used in a qPCR reaction to detect the presence of the WNV envelope E gene. WNV was detected in the blood on day 3 and the brain on day 7 of mice that received prM Ab-opsonized prMWNV but not in blood or brain from mice injected with prMWNV alone (Figure 3A/B). The day 3 blood and day 7 brain homogenates were also used to infect P388D1 macrophage-like cells. As expected, the mice that received prMWNV alone did not produce any infectious virus in their blood or brain. The mice that were infected with wt WNV and Ab-opsonized prMWNV had infectious virus in the blood on day 3 of infection and in the brain on day 7 of infection (Figure 3C). To confirm that the fluorescent signal seen in Figure 3C reflects production of infectious WNV virus and not only represents fluorescent staining of already infected cells from the blood and brain, we harvested the media from these cultures and used that to infect new P388D1cells. We were able to detect WNV infection in these cells via immunofluorescence staining using the antibody against WNV E protein conjugated with TRITC (Figure 3D). Taken together, these results confirm that mice infected with antibody-opsonized prMWNV particles died from a typical wt WNV infection.

A major risk factor for the development of severe dengue is the presence of pre-existing antibodies (Halstead, 2003). It is well accepted that antibodies target DENV particles to cells highly permissive to infection, leading to a higher number of infected cells and eventually increased disease burden (Halstead, 2003). This report shows for the first time that prM antibodies have the capacity to enhance the number of infectious particles present in vivo and strengthens the notion that immature particles and prM antibodies may increase flaviviral disease burden in humans.

Recent characterization of the humoral response of dengue patients with severe disease identified prM antibodies as the dominant fraction of the human antibody repertoire (Dejnirattisai *et al.*, 2010). Furthermore, Rai *et al.* showed a positive correlation between the circulating prM antibody titer and disease severity (Rai, 2008). These observations not only suggest that prM antibodies may be unfavorable for the host but also may have important repercussions for vaccine development. Generation of antibodies against the prM protein activates the infectious properties of immature DENV particles and therefore may have adverse effects on protection from infection. Future clinical studies are therefore required to probe the role of prM antibodies and immature particles in disease pathogenesis and to determine which types of antibodies are necessary for protection against disease.

ACKNOWLEDGEMENTS

This work was supported by the Pediatric Dengue Vaccine Initiative (JMS), NIH grants UO1 AI070343 and T32 AI07404 (EF), and by the Dutch Organization for Scientific research (NWO-ALW to JMS). EF is an investigator with the Howard Hughes Medical Institute.

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Enhancing effect of a fusion-loop antibody on the infectious properties of immature flavivirus particles

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Journal of Virology (2011). *Accepted for publication*

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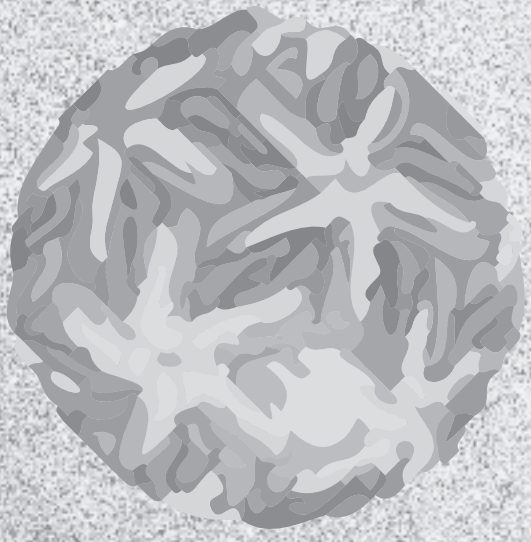

Chapter 7

ABSTRACT

Flavivirus-infected cells secrete a mixture of mature, partially immature, and fully immature particles into the extracellular space. Although mature virions are highly infectious, prM-containing fully immature virions are non-infectious largely because the prM protein inhibits the cell attachment and fusogenic properties of the virus. If, however, cell attachment and entry are facilitated by anti-prM antibodies, immature flavivirus virus become infectious after efficient processing of the prM protein by the endosomal protease furin. A recent study demonstrated that E53, a cross-reactive monoclonal antibody (MAb) that engages the highly conserved fusion loop peptide within the flavivirus envelope glycoprotein, preferentially binds to immature flavivirus particles. Here, we investigated the infectious potential of fully immature WNV and DENV particles opsonized with E53 MAb, and observed that like anti-prM antibodies, this anti-E antibody also has the capacity to render fully immature flavivirus particles infectious. E 53- mediated enhancement of both immature WNV and DENV depended on efficient cell entry and the enzymatic activity of the endosomal furin. Furthermore, we also observed that E53-opsonized immature DENV but not WNV particles required a more acidic pH for efficient cleavage of prM by furin, adding greater complexity to the dynamics of antibody-mediated infection of immature flavivirus virions.

Infectious properties of E53-opsonized immature flaviviruses

INTRODUCTION

Flaviviruses, including dengue virus (DENV serotypes 1, 2, 3, and 4) and West Nile virus (WNV), are small, enveloped, positive-strand RNA viruses that are transmitted to humans primarily by arthropods. On the flavivirus surface there are 180 copies of two transmembrane proteins: the major (51–60 kDa) envelope glycoprotein E, and the smaller (8 kDa) membrane protein M [14]. In the mature virion, the E glycoproteins are organized in 90 head-to-tail homodimers that lie flat on the viral surface. X-ray crystallography studies revealed that the ectodomain of each E monomer is comprised of three structural domains: DI, DII and DIII, connected by flexible hinges. The tip of DII contains a conserved region termed the "fusion loop", which is required for the low-pH-driven membrane fusion of the viral membrane with the host endosomal membrane [11,12,19,33].

Assembly of flavivirus particles occurs at the endoplasmic reticulum (ER) by formation of immature virions [15]. In immature particles, the E protein associates with prM, the precursor protein of M. The 90 E–prM heterodimers protrude from the viral envelope as 60 trimeric spikes. In this conformation, the pr peptide of the prM protein caps the fusion loop located at the distal end of each E monomer within the trimer [13,31,32]. Maturation of flaviviruses occurs during transit through the secretory pathway. In the mildly acidic lumen of the trans-Golgi network (TGN), the viral envelope proteins undergo low-pH driven conformational changes including dissociation of the prM-E heterodimers and formation of E homodimers [9,29]. Thereafter, the endoprotease furin cleaves the prM protein into a small M protein and a pr peptide. The pr peptide dissociates from the virion upon release of the particle to the extracellular milieu, which completes the formation of mature infectious virus [30].

The functional importance of flavivirus maturation has been investigated in significant detail. Multiple studies have shown that fully immature particles are non-infectious, with the presence of prM obstructing the low-pH-induced conformational changes in the viral E glycoprotein required for membrane fusion [7,9]. These observations led to the hypothesis that prM acts as a chaperone preventing premature fusion of progeny virions in the acidic compartments of the secretory pathway. Indeed, in vitro studies have shown that fusogenic activity of immature particles could be restored upon furin treatment, demonstrating that cleavage of prM to M is required to render flavivirions infectious [17,26,30,35].

We recently observed that fully immature particles become significantly infectious when opsonized with anti-prM monoclonal or serum antibodies. The prM antibodies facilitated efficient binding and entry of immature DENV into cells expressing Fcγ–receptors. Furthermore, furin activity within the target cell was required to render immature particles infectious indicating that immature particles undergo maturation after cell entry [23]. The ability of prM antibodies to rescue infectious properties of immature DENV was recently corroborated by observations of Dejnirattisai *et al*[5], using human MAbs.

In addition to antibodies against prM antibodies, those recognizing the E protein also can bind to immature virus particles. E53 is a fusion-loop-specific inhibitory anti-E MAb that preferentially binds to the immature form of WNV and DENV particles [3,21]. Consistent with this, E53 and other fusion-loop-specific MAbs neutralized partially mature (prM-containing) but not fully mature (prM-absent) WNV virions[18]. X-ray crystallographic analysis of E53 Fab

fragments complexed to WNV E protein have revealed that E53 engages 12 residues within the fusion peptide (G104, C105, G106, L107, G109, K110) and adjacent b-c loop (C74, P75, T76, M77, G78, E79) of DII. Fitting of the E53 Fab-WNV E crystal structure onto the cryo-EM structure of immature virions suggested that E53 may neutralize infection by impeding the transition from immature to mature virus by steric hindrance.

In this study, we investigated the influence of the E53 MAb on infectivity of fully immature DENV and WNV particles. Surprisingly, we observed that E53 significantly enhances the infectious properties of immature WNV particles. For immature DENV, enhancement of infection was observed in a cell type dependent manner. Whereas in Fc-receptor-expressing human erythroleukemic K562 cells no infectivity was observed, a marked increase in viral infectivity was seen in murine macrophage-like P338D1 cells. Analysis of the internalization pathway of E53 opsonized immature DENV particles suggested that this is related to a more acidic pH threshold for furin cleavage that is required to occur within endosomal compartments of the target cells. Furthermore, we show that in human peripheral blood mononuclear cells E53 mediated enhancement of wt DENV preparation is primarily dependent on the activity of furin. Overall, this report shows for the first time that in addition to anti-prM antibodies, those against E protein also can render immature flavivirus particles infectious.

MATERIALS AND METHODS

Cell culture.

C6/36 *Aedes albopictus* cells were maintained in minimal essential medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), 25 mM HEPES, 7.5% sodium bicarbonate, penicillin (100 U/ml), streptomycin (100 µg/ml), 200 mM glutamine and 100 µM nonessential amino acids at 28°C, 5% CO2. Baby hamster kidney-21 (BHK21) cells were cultured in DMEM (Invitrogen), supplemented with 5% FBS, 10% tryptose phosphate broth, 25 mM HEPES, 7.5% sodium bicarbonate, penicillin (100 U/ml), streptomycin (100 µg/ml) and 200 mM glutamine at 37°C, 5% CO2. BHK21 clone 15 cells (BHK21-15) were maintained in DMEM (Invitrogen), containing 10% FBS, 25 mM HEPES, 7.5% sodium bicarbonate, penicillin (100 U/ml), streptomycin (100 µg/ml), 10 mM HEPES and 200 mM glutamine. Human adenocarcinoma LoVo cells were cultured in Ham's medium (Invitrogen) supplemented with 20% FBS at 37°C, 5% CO2. Human erythroleukemic K562 cells were maintained in DMEM containing 10% FBS, penicillin (100 U/ml), and streptomycin (100 mg/ml) at 37°C, 5% CO2. Mouse macrophage-like P388D1 cells were maintained in DMEM supplemented with 10% FBS,penicillin (100 U/ml), and streptomycin (100 µg/ml), sodium bicarbonate (Invitrogen,7,5% solution) and 1.0 mM sodium pyruvate (GIBCO) at 37°C, 5% CO2. Human peripheral blood mononuclear cells (PBMCs) were maintained in RPMI 1640 medium supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 µg/ml). PBMCs were isolated from heparinized blood samples collected from healthy persons using standard density centrifugation procedures with Lymphoprep™ (AXIS-SHIELD). The PBMCs were used immediately after isolation or cryopreserved at -150°C. On the day of infection, the percentage of $CD14^+$, $CD19^-$ population within isolated PBMCs was determined (5 % -10 % depending on the blood donor) using cell surface markers CD-14 -FITC and CD19-R-PE purchased from commercial source (IQ Products).

Virus propagation.

DENV-2 strain 16681 was propagated in C6/36 cells as described previously [35]. WNV strain NY 385-99 (generous gift of Dr. J. Goudsmit, Crucell B.V., Leiden, The Netherlands) was propagated after inoculation of BHK21 cells at an MOI of 0.1. Culture medium was harvested at 48 hpi, cleared of cellular debris, aliquotted, and stored at -80°C. Fully immature DENV and WNV preparations were generated in LoVo cells as described previously [17,35]. [^{35}S] methionine-labeled immature virus preparations of WNV and DENV were prepared as described [17,35]. All virus preparations were analyzed with respect to the number of infectious (PFU) and genome-containing particles (GCPs) by plaque assay and quantitative (q)RT-PCR, respectively. For WNV, qRT-PCR analysis was performed using the

forward primer 5'-GTT GGC GGC TGT TTT CTT TC-3', the reverse primer 5'-GGG ATC TCC CAG AGC AGA ATT-3' and a TaqMan probe 5'-FAM-AAT GGC TTA TCA CGA TGC CCG CC-TAMRA-3' (Eurogentec, Seraing, Belgium). DNA was amplified for 40 cycles (15 s at 95°C and 60 s at 60°C) on a StepOne real-time PCR instrument (Applied Biosystems, Carlsbad, CA) and the number of copies of WNV RNA was quantified using a standard curve based on a cDNA plasmid containing the non-structural genes of WNV NY99 (kind gift from Dr. G.P. Pijlman, Wageningen University, The Netherlands).

ELISA.
The reactivity of MAb E53 to immature DENV or WNV was determined by standard three-layer ELISA with a horseradish peroxidase-based detection system as described previously [23], In the experiments, wherein the effect of the low pH on the binding of E53 to immature virions was evaluated, additional 15-min acidic (pH 5.0, 5.5, 6.0, 6.5), washes were introduced.

Infectivity assays.
Virus or preformed virus-MAb complexes were incubated with K562 or P388D1 (2×10^5) cells at a multiplicity of 10, 100 or 1000 GCPs (MOG) per well for WNV and DENV, respectively. At 26 (WNV) or 43 (DENV) hpi, medium was harvested and virus yield was analyzed by plaque assay on BHK21-15 cells, as described previously [6]. Virus-MAb complexes were formed by incubating virus for 1 h at 37°C with increasing concentrations of MAb E53 in cell culture medium containing 2% FBS prior to the addition to cells. The DENV anti-prM mAb 70-21 was included as a positive control 23. In furin blockade experiments, the cells were treated with 25 μM of the furin-specific inhibitor decanoyl-L-arginyl-L-valyl-L-lysyl-L-arginyl-chloromethylketone (decRRVKR-CMK, Calbiochem, Darmstadt, Germany) prior to and during infection.

Binding and cell Internalization assays.
To determine the number of bound/internalized viruses per cell, virus or virus-MAb complexes were incubated with K562 (2×10^5) cells at MOG 1000 for 1 h at 37°C as described previously [23]. Subsequently, cells were washed extensively with PBS containing $MgCl_2$ and CaCl to remove unbound virus-MAb complexes. To quantify internalized virions, cells were treated for 1.5 h with 0.5mg of proteinase K (Invitrogen). Viral RNA was extracted from cells and from control cells' washes using the QIAamp Viral RNA mini kit (QIAGEN, Valencia, CA). cDNA was synthesized from the viral RNA with reverse-transcription PCR (RT-PCR), and copies were quantified using qRT-PCR analysis.

In vitro furin cleavage assay.
[^{35}S]methionine-labeled immature particles or viral immune-complexes were incubated with furin (New England Biolabs, Ipswich, MA) for 16 h at pH 6.0, as described previously [65,66] or at a specified pH as indicated in the Results. Following furin treatment, viral proteins were visualized by subjecting the samples to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and phoshorimaging analysis on a Cyclone scanner (Perkin Elmer, Waltham, MA).

RESULTS

E53 renders immature WNV and DENV particles infectious in a cell-dependent manner.

The infectious properties of immature WNV and DENV particles opsonized with increasing amounts of the fusion loop cross-reactive MAb E53 were investigated in two Fcγ-receptor-expressing cell lines, human leukemia K562 cells and murine P388D1 macrophages. To this end, we first generated immature WNV and DENV particles in furin-deficient LoVo cells using a published protocol [17,35]. The specific infectivity of the LoVo cell-derived virus used in this study was reduced greater than 10,000-fold compared to that of the st virus preparation, in agreement with our previous data [17,35]. After these initial characterizations, K562 and P388D1 cells were infected with LoVo cell-derived WNV and DENV particles in the presence of increasing concentrations of E53. At 26 (WNV) or 43 (DENV) hours post-infection (hpi), the (infectious) viral titer in the supernatant was determined by plaque assay. Interestingly,

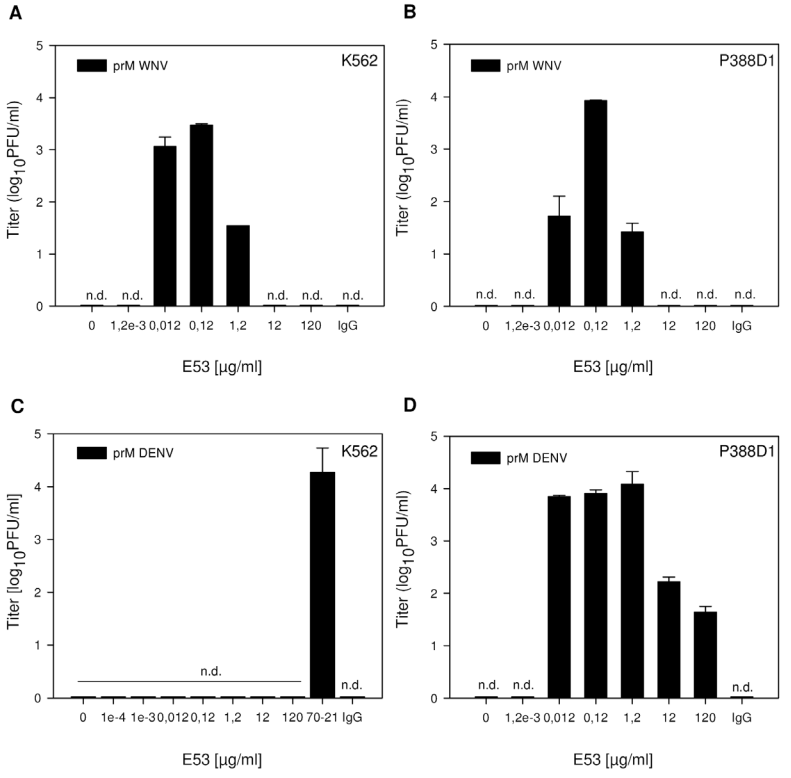

Figure 1. E53 renders immature WNV and DENV particles infectious in cell dependent manner.
Immature WNV (A, B) and DENV (C, D) particles were incubated with increasing concentrations of E53 for 1 h at 37°C. For DENV, the prM antibody 70-21 (40 ng/ml) was included as a positive control. K562 cells were infected at MOG 10 and 100 for WNV and DENV, respectively. At 26 (WNV) and 43 (DENV) hpi, virus production was measured by plaque assay on BHK21-15 cells. Data are expressed as the mean of at least three independent experiments. Error bars represent standard deviations (SD); (n.d.) denotes "not detectable".

coating of immature WNV particles with the E53 MAb significantly stimulated viral infectivity in both K562 and P388D1 cells (Fig. 1A and B, $P < 0.0001$). At an E53 concentration of 0.012 and 0.12 µg/ml in K562 cells and 0.12 µg/ml in P388D1 cells greater than a 1,000 fold enhancement of infectious WNV production was observed. At higher MAb concentrations, neutralization of infection was seen. An E53 antibody concentration of 0.12 µg/ml corresponds to 8×10^{-10} M and addition of 2×10^4 MAb molecules per virion, indicating that a large excess of antibody is required to trigger infectivity. Surprisingly, whereas E53 did promote infectivity of immature DENV in P388D1 cells (Fig. 1D, $P < 0.0001$), over a broad range of antibody concentrations, no infectivity was observed in K562 cells, while the anti-prM mAb 70-21 did stimulate infectivity of immature DENV in these cells (Fig. 1C, ref. 23).

The lack of infectivity of E53-opsonized immature DENV in K562 cells is not caused by impaired virus internalization.

The observed difference in the enhancing effect of E53 in K562 vs. P338D1 cells between WNV and DENV prompted us to investigate the entry of immature flavivirus particles in these

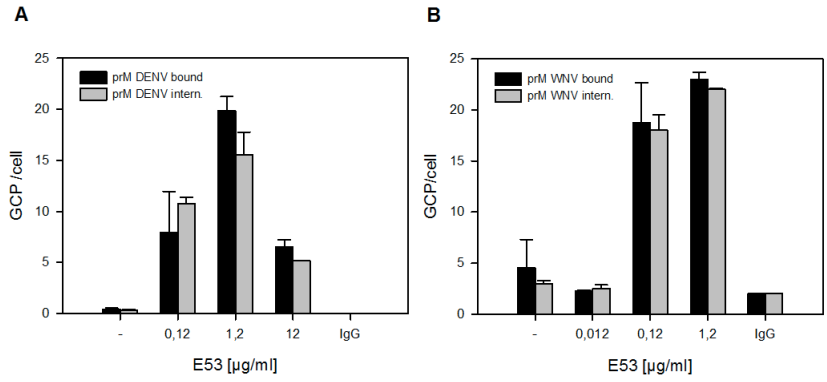

Figure 2. E53 facilitates efficient binding and cell entry of immature WNV and DENV.
E53-coated immature DENV (A) and WNV (B) particles were incubated with K562 cells at MOG 1,000 for 1 h at 37°C. Unbound virus was washed away and virus associated with K562 cells was detected by qRT-PCR analysis. Internalization was assessed after removal of the bound virus with proteinase K treatment. Data are expressed as the mean of at least three independent experiments performed in duplicates. Error bars represent standard deviations (SD); (n.d.) denotes "not detectable".

cells. While the observation showing that E53-opsonized immature WNV is infectious in K562 cells suggests that E53 facilitates efficient entry of immature flavivirus particles into K562 cells, we assessed whether this was also true for immature DENV virions. Immature DENV and WNV particles were pre-incubated with increasing concentrations of E53 MAb and added to K562 cells for 1 hr at 37°C to allow cell binding and internalization. After extensive washing, the number of bound or, after treatment with proteinase K, internalized virions per cell was determined by quantitative RT-PCR. To reliably determine the number of bound GCPs per cell, the amount of virus added per cell was increased 10-fold compared to the concentration used in the infectivity experiments. Independent experiments showed that the higher concentration of input virus did not affect viral infectivity of immature virus by E53 (data not shown). As expected, in the absence of E53 MAb or in the presence of control MAb, virtually no cell binding was observed for immature WNV or DENV, confirming that immature particles fail to interact efficiently with K562 cells (ref. 23 and Fig. 2) Notably, E53 facilitated efficient binding and cell entry of not only immature WNV but also immature DENV, demonstrating that the lack of infectivity observed for E53-opsonized immature DENV is not related to inefficient internalization to K562 cells.

Furin protease activity is required to render immature flavivirus particles infectious

Given the binding and internalization findings in K562 cells, we next assessed the role of furin during cell entry as its protease activity in cells is crucial for rendering immature DENV particles opsonized by prM antibodies infectious [23]. We treated K562 cells and P388D1 cells prior to and during infection with the furin inhibitor, decanoyl-L-arginyl-L-valyl-L-lysyl-L-arginyl-chloromethylketone (decRRVKR-CMK). The half-life of decRRVKR-CMK is 4 to 8 h. Indeed, we previously reported that this treatment does not interfere with the formation of infectious particles following infection with st DENV [23]. Furin inhibitor was observed to only affect virus particle maturation upon addition of the compound at the moment of virion assembly [23]. As shown in Figure 3, addition of furin inhibitor at the time of infection does not influence ma-

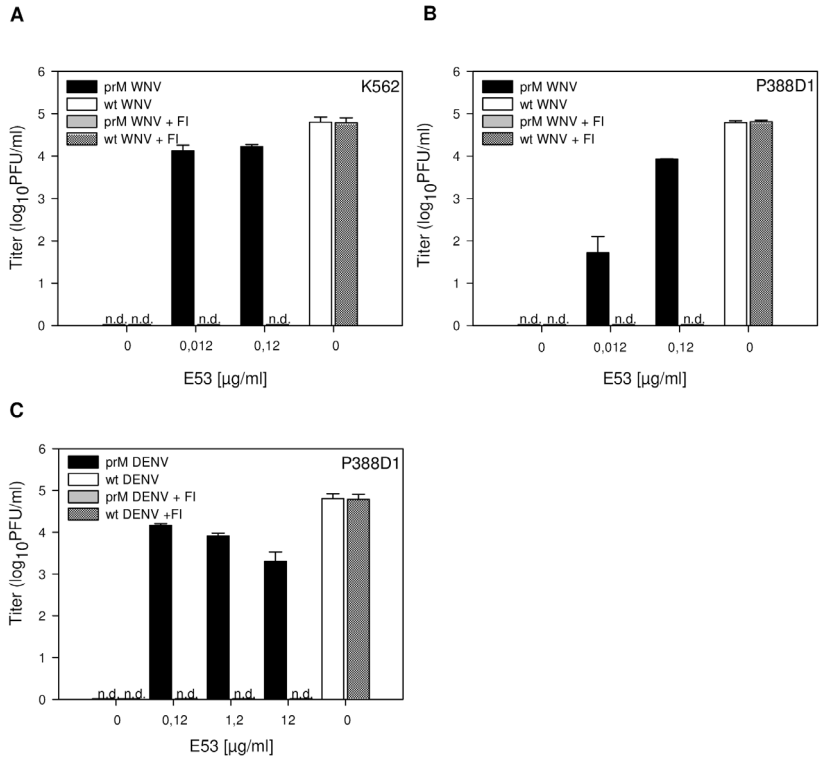

Figure 3. Furin protease activity is essential for the infectivity of immature flaviviruses particles.
For furin blockade, K562 (A) and P388D1 (B and C) cells were treated with 25 µM of decRRVKR-CMK prior to, and during infection with E53-coated immature WNV and or DENV. Virus production was assessed as described in the legend of Figure 2. Data are expressed as mean of at least two independent experiments performed in triplicate. Error bars represent standard deviations (SD) of the cumulate six datapoints, (n.d.) denotes "not detectable".

turation of newly assembled virions within infected cells. Importantly, the infectivity of E53-opsonized immature WNV (Fig. 3A and 3B for K562 and P388D1 respectively) and DENV particles in PD388D1 (Fig. 3C) was lost in the presence of the inhibitor, and thus, required the enzymatic activity of furin. These results confirm that furin cleavage of prM to M is a prerequisite step in the cell entry process of antibody-coated immature flavivirus particles, regardless of whether the enhancing antibody is directed at the prM or E proteins.

E53 affects the cleavage of DENV prM by furin

Because recent structural analysis suggested that E53 neutralizes infection by impeding the transition from immature to mature virus [3], we hypothesized that the lack of infectivity of E53-bound immature DENV particles in K562 cells was related to the inability of furin to cleave prM to M, possibly due to steric hindrance. To evaluate this, we incubated [^{35}S]methionine-labeled immature WNV and DENV in the absence and presence of E53 MAb with exogenous furin at pH 6.0, the condition that mimics the mildly acidic milieu of the early endosomal lumen. Subsequently, the protein composition of the virus particles was analyzed by SDS–PAGE and phosphorimaging. Whereas efficient cleavage of prM to M was observed for WNV particles in the presence of E53 and furin (Fig 4A), prM processing was completely inhibited under the same conditions for immature DENV particles (Fig. 4B). The ability of furin to cleave E53-

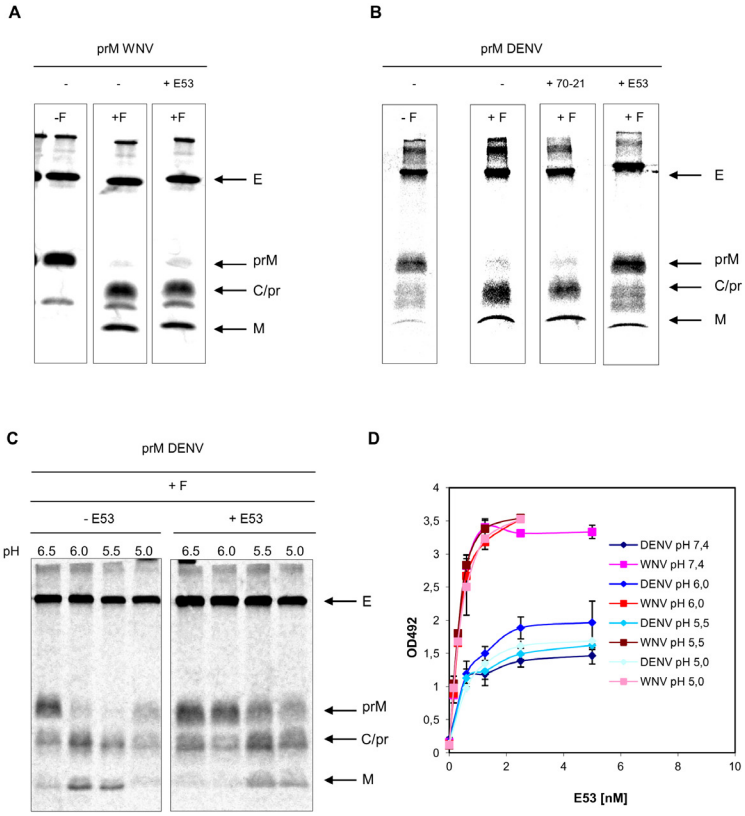

Figure 4. Influence of E53 MAb on the cleavage of immature flaviviruses by furin.
[^{35}S]methionine-labeled immature virus was incubated with increasing amounts of MAb E53 for 1 h at 37°C. Subsequently, virus-MAb complexes were subjected to furin cleavage in vitro at pH 6.0 or at decreasing pH values for 16 h. The viral protein composition was then analyzed by SDS-PAGE and phosphorimaging analysis. E53 does not impair prM to M cleavage of immature WNV (A) but blocks that of immature DENV (B). Protein composition of furin-cleaved immature WNV opsonized with 0.12 µg/ml E53 and immature DENV opsonized with 0.12 µg/ml E53, immature DENV opsonized with 0.4 µg/ml mAb 70-21 was used as a control (70-21 lanes are adapted from ref. 23 as these were analyzed simultaneously). (C) The presence of E53 alters the cleavage of immature DENV by furin in a pH-dependent manner. Protein composition of immature DENV opsonized with 0.12 µg/ml and cleaved with furin is solution at various acidic pH. Data is representative of at least two independent experiments. (D) Effect of the low pH on the avidity of E53 mAb binding to immature flaviviruses was measured by direct ELISA. Background OD values were subtracted from the data points to show only virus-specific signal. Data were analyzed as described in the Materials & Methods, error bars represent the standard errors of data from duplicate wells of at least 3 experiments.

opsonized immature WNV was observed over a wide antibody concentration range (0.3 nM to 300 nM) including conditions at which enhanced virus-cell binding and entry was observed (results not shown); under none of these conditions cleavage of DENV prM to M detected.

While blockade of furin cleavage by E53 antibody explained the lack of enhancement of immature DENV in K562 cells, it was not consistent with the observed infectivity of antibody-bound immature DENV in P388D1 cells. Because endosomal pH can vary substantially between different cells [2,24], and flaviviruses undergo low-pH-triggered structural changes prompting dissociation of prM/E heterodimers, we hypothesized that E53 might bind to virus particles in a manner that impairs furin cleavage at mildly acidic pH, yet allowing furin cleavage at lower pH values. Initially, we attempted to examine the cleavage status of the virus after cell

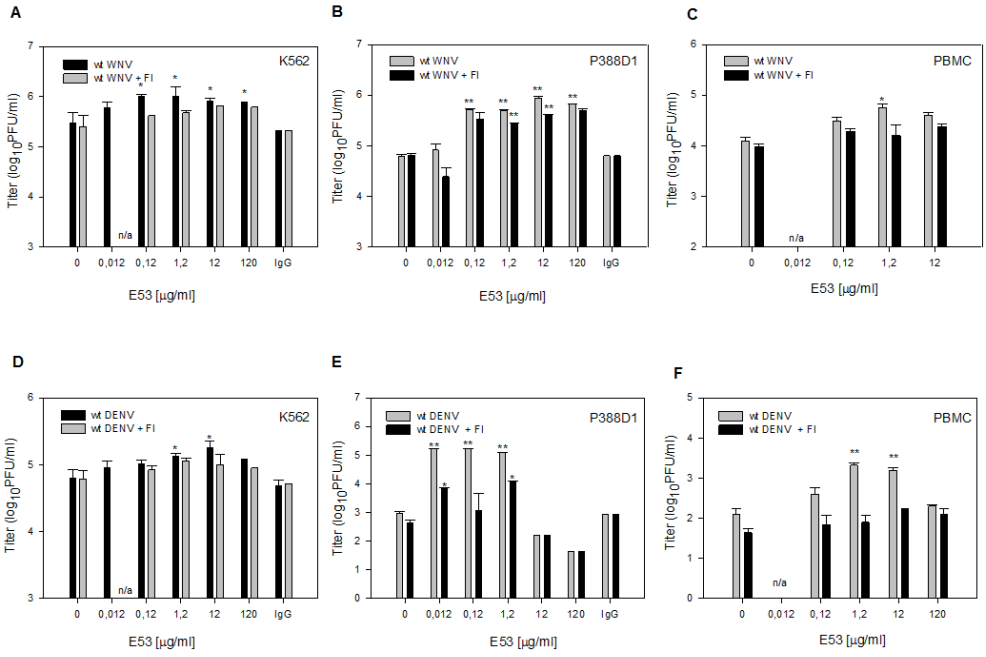

Figure 5. Enhanced infectivity of wild-type WNV and DENV after incubation with E53.
St WNV (A, B, C) and st DENV (D, E, F) particles were incubated with increasing concentrations of E53 for 1 h at 37°C. K562 cells (panels A and D), P388D1 cells (panels B and E) and PBMC's (panels C and F) were infected at MOG 10 and 100 for WNV and DENV, respectively. For furin blockade, K562 and P388D1 cells were treated with 25 µM of decRRVKR-CMK as described in the legend of Figure 3. Virus production was assessed as described in the legend of Figure 1. Data are expressed as mean of at least three experiments. Error bars represent standard deviations (SD). ** $p < 0.001$, * $p < 0.05$.

entry in K562 cells but due to the low number of infected K562 cells even at high multiplicities of infection this could not be defined. As a surrogate model, we performed the same *in vitro* furin cleavage experiment as described above but now under different pH conditions. Immature DENV was incubated with HNE buffer containing E53 antibody or with the buffer alone and subsequently subjected to the furin cleavage experiment at pH 6.0; 6.0; 5.5, 5.0. Remarkably, E53 shifted the pH threshold for furin cleavage of DENV immature particles to more acidic values, indicating that antibodies can directly modulate the pH-dependent structural changes after entry (Fig. 4C).

Based on these results we hypothesized that E53 stays associated with immature DENV at mildly acid pH thereby preventing furin cleavage and infection and dissociates from the virus at lower pH values allowing furin cleavage and subsequent infection. To test this, we performed ELISA experiments in which low pH (range 6.5 to 5.0) washes were performed following the incubation with E53. Consistent with prior studies [3,21], we observed that E53 efficiently binds to immature WNV and DENV particles at neutral pH. In contrast to our hypothesis, we found that E53 binding to immature particles is not pH-dependent (Fig. 4D), which suggests that dissociation of E53 from the immature virion is not a prerequisite for furin cleavage. We now therefore propose, although difficult to substantiate, that exposure of E53-opsonized DENV to lower pH values is necessary to overcome the energy threshold that is required to induce the global rearrangement of the virion prior to furin cleavage.

Furin activity is important for E53-mediated enhancement of the standard flavivirus preparations

Standard flavivirus preparations contain a mixture of mature, immature and partially mature virions, the latter containing a mixture of prM and M [3,10,17,18,27,35]. Indeed, a substantial fraction of DENV particles secreted from C6/36 insect cells are partially mature [3,10]. Since furin-activity is crucial to render fully immature virions infectious, we investigated whether E53, which preferentially binds to the immature virions, enhances infectivity of st virus preparations in a furin-dependent manner. We performed the enhancement assays in K562 cells (Fig. 6A/D), P388D1 cells (Fig. 5B/E) as well as in human PBMCs (Fig. 5C/F). Notably, E53 enhanced the infectivity of both st WNV preparations (Fig. 5A-C) and DENV (Fig. 5D-F). The level of enhancement for st WNV in K562 cells (2- to 5-fold, $P < 0.05$) and PBMCs (2-to 5-fold, $P<0.05$) was slightly less than in P388D1 cells (4- to 10-fold, $P < 0.001$). Interestingly, E53 also enhanced infection of st DENV in K562 cells (2- to 3-fold, $P<0.05$, Fig. 5D) albeit to a lesser extent than in P388D1 cells (70- to 200-fold, $P< 0.001$, Fig. 5E) or PBMCs (10- to 20-fold, $P < 0.001$, Fig. 5F). The enhanced infectivity of E53-opsonized st DENV in K562 cells was not dependent on furin activity, in agreement with the observation that E53-opsonized immature virions were not infectious in these cells. In comparison, the higher degree of furin-dependent enhancement seen for E53-opsonized st DENV compared to WNV in P388D1 and PBMCs cells suggests that DENV produced in insect cells contains a higher fraction of partially immature and fully immature particles (those requiring maturation upon entry) than WNV. This may also explain why a higher level of enhancement was seen in P388D1 cells and PBMCs compared to K562 cells, since furin cleavage of E53-opsonized immature DENV is likely inhibited in K562 cells.

DISCUSSION

In this study, we show for the first time that the flavivirus cross-reactive MAb E53, which maps primarily to the fusion-loop in DII, can render fully immature flavivirus particles infectious. Enhancement of infection by E53-opsonized immature DENV was cell type-specific since E53 stimulated infectivity of immature DENV particles in a murine macrophage cell line, but did not enhance infection in human K562 cells. E53 facilitated efficient binding and cell entry of both immature DENV and WNV in K562 cells, and E53-mediated enhancement of infection of immature virions was strictly dependent on furin activity in the target cell. Analysis of the pH-dependent furin cleavage step revealed that E53 inhibits prM cleavage of immature DENV particles at a mildly acidic pH values, but that this can be overcome at lower pH. This suggests that the ability of antibodies to stimulate infectivity of immature DENV is cell type-dependent, presumably due to the unique host environment, including the pH of endosomes, after virus entry. The prerequisite of furin activity for E53-mediated enhancement of wt DENV, but not that of immature WNV, not only substantiates the difference between these two flaviviruses, but also underlines the potentially important role of immature or partially mature DENV in antibody-dependent enhancement of infection.

The observation that anti-E antibodies can render immature flavivirus particles infectious is novel and has implications for our understanding of the mechanisms of virus cell entry. Within

acidified endosomes, the immature virion undergoes a major structural rearrangement, allowing furin to cleave prM to M and a pr peptide. Recent data suggest that the pr peptide remains associated with the particle at pH 5.5 [30]. The pr peptide is believed to protect newly assembled virions from adventitious fusion during transit through the acidic trans-Golgi network, and to be released only after the particle reaches the extracellular milieu, which has a slightly basic pH. However, it remains unknown how the pr peptide is released after furin cleavage of immature particles within endosomes. We have previously hypothesized that following furin cleavage the pr peptide will be released from the particle due to its interaction with prM antibodies thereby enabling the E proteins to undergo the conformational change required for fusion [23]. Here, we show that an anti-E MAb also stimulates the infectivity of immature virions. Thus, it seems more plausible that dissociation of the pr peptide from the virion can be triggered directly by specific conditions in late endosomes, such as e.g. the lower pH (~5.0) environment. This notion is supported by a recent study demonstrating that inhibition of fusion by pr peptide is less efficient in this lower pH range [34]. Alternatively, cleaved immature particles may be recycled with or without antibody back to the plasma membrane and/or extracellular space to allow pr dissociation and subsequent initiation of infection upon re-entry of the virions into the endocytic pathway.

Previous structural studies have suggested that the fusion-loop MAb E53 may block the transition of immature to mature particles by steric hindrance [3]. The results presented here confirm that E53 does inhibit cleavage of prM to M of fully immature DENV particles under mildly acidic pH values. By contrast, E53-opsonized immature WNV particles were processed efficiently under the same conditions. These data may explain, at least in part, a prior observation that fusion loop-specific MAbs that were generated against WNV (e.g., MAbs E18, E53, or E60) had far greater neutralizing activity against DENV infection [1,18,21] and why they blocked WNV infection primarily at a stage of viral attachment [20] rather than fusion as seen with DENV [4]. Interestingly the neutralizing effect of E53 on the immature DENV particle was pH-dependent and cell type-specific, suggesting that depending on the cellular context, opsonized immature dengue particles may be neutralized or rescued. Thus, for DENV, the relative pH in the early endosomes presumably controls the fate of E53-opsonized immature virus. On the other hand, in K562 cells, which are reported to have a low early endosomal pH[25,28], immature DENV is neutralized, suggesting that optimum pH for furin cleavage is not the sole prerequisite for the immature virus to gain infectivity. From a structural perspective, it remains unclear why E53 has a distinct effect on immature WNV and DENV particles. Because the one amino acid difference (residue E77: DENV Q, WNV M) in the E53 structural epitope on WNV and DENV represents a polar amino acid, it is tempting to speculate that, at mildly acidic pH, E53 stabilizes the viral spike complex of immature DENV to such an extent that the global conformational changes are blocked whereas in case of immature WNV E53 does not prevent the conformational change and furin cleavage to occur.

The humoral response generally has a crucial function in controlling flavivirus infections [22]. However, for DENV, antibodies are not only involved in viral clearance, but - under certain conditions - may also be associated with development of severe disease symptoms by so-called antibody-mediated enhancement (ADE) of infection. The ADE hypothesis suggests that at

sub-neutralizing concentrations antibodies will target virions to Fcγ-receptor-bearing cells and, thus, expand the number of infected cells and consequently the viral load [8]. Recent studies suggest that the ability of an antibody to neutralize or enhance infection may be modulated by the maturation state of a virus particle [18,23]. Specifically, antibodies against prM [5,23] and, as demonstrated in the present study, the fusion-loop on E can render non-infectious fully immature particles infectious. The hallmark of these antibodies is that they are generally cross-reactive between DENV serotypes and poorly neutralizing [5,18]. In addition, these antibodies bind avidly to immature or partially mature virions, and thus may target these virus particles efficiently to Fcγ-receptor-bearing cells, underlining the potentially important role of immature or partially mature DENV in ADE and the pathogenesis of severe disease. Accordingly, the presence of (partially) immature virions and anti-E or anti-prM antibodies, which preferentially or solely interact with the immature aspect of these virus particles, may well be of particular importance for immune enhancement during secondary heterosubtypic DENV infection

Based on our prior studies with anti-prM antibodies and those described here, we suggest that not only efficient entry but also particle maturation within the target cell represent important steps in antibody-mediated enhancement of DENV infection. Clearly, in the case of fully immature virus, furin-mediated maturation within the target cell is essential for rescue of viral infectivity. On the other hand, for st virus, which in addition to fully mature and immature virus also contains partially mature particles, furin cleavage is not essential since the mature aspect of these partially mature virions may undergo the low-pH-induced conformational change without additional cleavage of prM and release of pr peptide. Consistent with this model, E53 did stimulate the infectivity of st DENV in K562 cells, while these cells do not support furin cleavage of prM. However, in P388D1 cells, which do support virions maturation, E53-dependent enhancement of st DENV infection was much more pronounced than in K562 cells, indicating that cleavage of prM within the target cell significantly contributes to the enhancement of infection. Also, in human PBMC, which comprise major DENV target cells, E53-mediated enhancement of st DENV infection was strongly dependent on furin activity, again indicating that furin-mediated maturation, also in these physiologically important cells, is an important factor in antibody-mediated enhancement of infection.

Not only antibody-mediated enhancement, but also antibody neutralization of infection depends on the maturation status of the virion. Maturation of WNV particles reduced the ability of fusion-loop antibodies, such as E53, to neutralize infection [18]. Given that fusion-loop MAbs preferentially recognize immature particles [3] the decreased neutralizing activity of E53 with fully mature virions has been explained by the hypothesis that binding does not reach an occupancy sufficient for neutralization [22]. Consistent with this notion, addition of the complement component C1q, which reduces the stoichiometric threshold of antibody neutralization, allowed E53 to neutralize fully mature WNV more efficiently [16]. Based on these prior studies and the data presented here, it is now clear that the structural architecture of individual viral particles influences the outcome of infection. Future studies that define more precisely the biochemical and cell-biological mechanisms of antibody-mediated neutralization or enhancement of immature virus particles will undoubtedly clarify flavivirus disease pathogenesis and promote efforts for safe and effective vaccine development.

Chapter 7

ACKNOWLEDGMENTS

This work was supported by the Pediatric Dengue Vaccine Initiative, the National Institute of Health (R01-AI077955), and by the Dutch Organization for Scientific research (NWO-Earth and Life Sciences).

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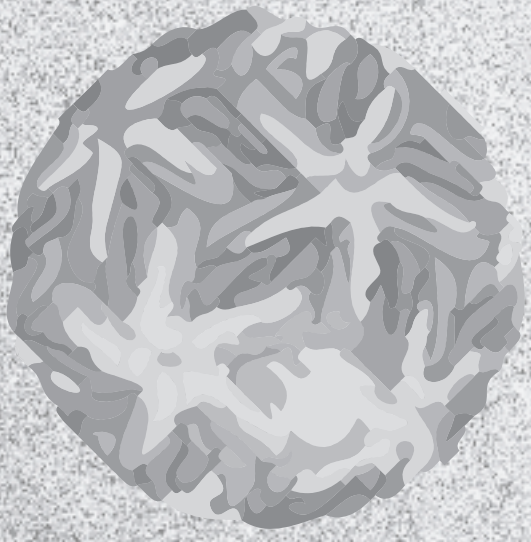
Summarizing discussion



Chapter 8

INTRODUCTION

Antibodies (Abs) are critical in the protection against and clearance of flavivirus infections (Pan *et al.*, 2001; Diamond *et al.*, 2003; Shrestha *et al.*, 2008). Indeed, in murine models for flavivirus infection, passive transfer of monoclonal antibodies (MAbs) or immune serum protects mice against lethal challenge (Jacoby *et al.*, 1980; Mathews & Roehrig, 1984; Diamond *et al.*, 2003; Goncalvez *et al.*, 2008). Furthermore, several MAbs have been shown to have therapeutic potential in mice when administered several days after otherwise lethal infection, highlighting their importance in clearing the virus from peripheral organs (Kimura-Kuroda & Yasui, 1988; Roehrig *et al.*, 2001; Oliphant *et al.*, 2005; Goncalvez *et al.*, 2008; Shrestha *et al.*, 2010; Sukupolvi-Petty *et al.*, 2010). The Ab response to flavivirus infection is directed against the structural glycoproteins prM and E, and the nonstructural protein 1 (NS-1) (Throsby *et al.*, 2006; Lai *et al.*, 2008; Dejnirattisai *et al.*, 2010). Interestingly, the ability of Abs directed against the E-glycoprotein to potently inhibit viral entry into target cells was found to be an important correlate of protection *in vivo* (Oliphant *et al.*, 2005; Throsby *et al.*, 2006; Oliphant *et al.*, 2006; Shrestha *et al.*, 2010). At the same time, enhanced uptake of E Ab-opsonized flaviviruses into cells bearing Fc-*y* receptors (FcR) has been proposed to be directly involved in DENV pathogenesis through a mechanism known as Ab-dependent enhancement (ADE) of infection (Halstead & O'Rourke, 1977; Halstead *et al.*, 1977; Halstead, 2003; Dejnirattisai *et al.*, 2010; Balsitis *et al.*, 2010). Consequently, a more detailed insight into the entry mechanisms of flaviviruses is crucial for our understanding of antibody-mediated neutralization and enhancement of flavivirus infection. The studies described in this thesis focus on the various steps in the cellular entry pathways of DENV and WNV as important targets for Ab-mediated neutralization or enhancement of flavivirus infection.

STRUCTURAL TRANSITIONS IN THE FLAVIVIRUS LIFECYCLE

Flavivirus particles are dynamic structures. The viral surface proteins undergo large-scale conformational rearrangements during virus assembly and maturation, and their subsequent entry into target cells. In mature virus particles, 90 E-glycoprotein homodimers are orientated perpendicular to the viral surface in a head-to-tail fashion (Kuhn *et al.*, 2002; Zhang *et al.*, 2004). The E-protein itself consists of three domains designated DI – III (Rey *et al.*, 1995; Modis *et al.*, 2003; Nybakken *et al.*, 2006; Kanai *et al.*, 2006). DI consists of an 8-stranded β-barrel and bridges the other two domains through flexible linkers, providing flexibility to the E-protein. Protruding from DI is a finger-like domain termed DII, with the fusion loop (cd-loop) at its distal end. Its extended β-stranded structure is crucial for oligomeric interactions between E-proteins. DIII is an Ig-like domain and anchors the E-protein into the viral membrane through a stem region. In addition, DIII is postulated to contain the receptor-binding domain (Rey *et al.*, 1995; Chen *et al.*, 1997; Lee & Lobigs, 2000).

Upon binding of the E-glycoprotein to the cell surface, the flavivirus particles are internalized through clathrin-mediated endocytosis (Chu & Ng, 2004; van der Schaar *et al.*, 2008) and fuse from within acidic endosomes, thereby releasing its RNA genome into the cell cytosol (van der Schaar *et al.*, 2007; Krishnan *et al.*, 2007; van der Schaar *et al.*, 2008; Sips, Moesker, Wil-

schut & Smit, unpublished results). In **Chapter 3** we investigated the functional requirements for West Nile virus (WNV) membrane fusion using liposomal target membranes. We found that mildly acidic pH is the sole condition required to trigger fusion with liposomes, which is in accordance with earlier studies on WNV and tick-borne encephalitis virus (TBEV) (Gollins & Porterfield, 1986b; Corver *et al.*, 2000). The architecture of the E-protein has evolved to respond to changes in pH through a sensoring mechanism that likely involves one or more highly conserved histidine (His) residues (Kampmann *et al.*, 2006; Fritz *et al.*, 2008). Protonation of these His residues, and possibly other residues with ionizable side-chains (Nelson *et al.*, 2009) serves as a 'pH switch' that promotes a series of conformational rearrangements of the E-protein lattice on the virion surface. The meta-stable dimeric conformation of the E-glycoprotein is destabilized upon exposure to low pH, causing the dimers to dissociate (Stiasny *et al.*, 1996; Stiasny *et al.*, 2002; Stiasny *et al.*, 2007). Through a monomer intermediate, the E-protein lattice on the viral surface then rearranges into 60 homotrimers (Bressanelli *et al.*, 2004; Modis *et al.*, 2004). During this highly orchestrated molecular event, the fusion loops at the tips of the extended monomers are inserted into the target membrane. Refolding of the monomers into trimers allows for DIII and the stem region within each monomer to 'zipper up' alongside the trimer interface to form a hairpin (Bressanelli *et al.*, 2004; Stiasny *et al.*, 2005; Liao *et al.*, 2010), thereby bringing the viral membrane and target membrane into close proximity. At this point, the outer layers of the two lipid membranes will merge, forming a so-called hemifusion intermediate. The cooperative association of increasing numbers of activated trimers is believed to be required for progression into a complete fusion pore (Gibbons *et al.*, 2004). This notion is supported by experimental observations suggesting that the most energy-intensive stages in the fusion process follow rather then preceede the formation of a hemifusion intermediate (Zaitseva *et al.*, 2005).

As flavivirus particles progress through the endocytic pathway they encounter increasingly lower pH values. In **Chapter 3**, we show that WNV membrane fusion is most optimal (in terms of the rate and extent of fusion) at pH 6.3. At higher pH values, lower rates and extents of fusion were observed with a pH threshold for fusion of approximately 6.9. The observation that, in our lipid-mixing assay, a smaller fraction of the virus population undergoes fusion at pH 6.7 compared to pH 6.3, suggests that some particles fuse at pH 6.7 while others do not. This apparent heterogeneity may relate to the maturation state of individual particles (as will be discussed below) and their ability to 'recruite' activated trimers to participate in the formation of fusion pores at suboptimal pH values (Zaitseva *et al.*, 2005). It would be of interest to investigate the fusion kinetics of WNV particles at these pH values at a single-particle level (Floyd *et al.*, 2008). One possible outcome of such a study could be that particles unable to fuse at pH 6.7 would undergo fusion upon further acidification to pH 6.3. This would be in line with a recent study in DENV suggesting that flavivirus fusion within living cells is very efficient, with 1 out of 6 particles that bind to the cell eventually undergoing membrane fusion (van der Schaar *et al.*, 2007).

Major rearrangements of the viral glycoproteins also play an important role in the assembly and maturation of flavivirus particles. The morphogenesis of flavivirus particles is initiated when newly formed nucleocapsids containing the viral genome bud into the endoplasmatic

reticulum (ER) and are enclosed by a lipid bilayer anchoring the viral transmembrane proteins prM and E (Mackenzie & Westaway, 2001; Lorenz *et al.*, 2002; Lindenbach & Rice, 2003). Within the resulting immature virions, the prM and E proteins are associated as heterodimers which in turn form 60 trimers that protrude from the virion surface, giving immature particles a "spiky" appearance (Zhang *et al.*, 2003; Zhang *et al.*, 2007). Analysis of the X-ray crystallographic structure of a recombinant DENV prM-E fusion protein has provided some insight into the mechanism by which the flavivirus particle is stabilized by the prM protein (Li *et al.*, 2008). Like the fusion process, maturation too is highly regulated in a pH-dependent manner. Within the heterodimeric conformation, the M polypeptide portion of the prM protein is extended alongside DII of the E-protein, with the pr-portion covering the fusion loop at its distal end. The virus particles mature during transit through the Golgi apparatus. Upon exposure to the low pH conditions of the Trans Golgi network (TGN), the particle adopts a conformation in which the E-proteins are arranged as homodimers, analogous to the smooth prefusion conformation of the mature particle. This structural rearrangement allows for cleavage of the prM protein by the host endoprotease furin. Importantly, the pr-peptide remains associated with the fusion loop through electrostatic interactions until the particle is exposed to neutral pH (Yu *et al.*, 2008). Dissociation of the pr-peptide after exocytosis primes the particle to undergo membrane fusion upon subsequent exposure to low pH. Indeed, in **Chapter 3**, we observed that fully immature WNV particles do not undergo membrane fusion with liposomes when exposed to pH values of 6.3 or lower. Furthermore, in line with the mechanism proposed by Li and coworkers (Li *et al.*, 2008), *in vitro* maturation of immature virus by exposure to pH 6.0 in the presence of furin followed by back-neutralization, fully activated the fusogenic properties of immature WNV particles (**Chapter 3**, Fig. 3).

Interestingly, the flavivirus maturation process is not very efficient. We have shown that the protein composition of wildtype preparations of WNV or DENV grown on BHK-21 or C6/36 cells respectively, contains on average ~30% prM (Zybert *et al.*, 2008; **Chapter 3**). In a recent study, an attempt was made to investigate the distribution of prM on a per particle basis using sequential immunoprecipitation (Junjhon *et al.*, 2010). It was found that extracellular virus consists of three subpopulations of virus: fully mature (containing only M), fully immature (containing only prM) and partially immature (containing both M and prM). This experimental evidence is supported by cryo-electron microscopic (cryoEM) observations of particles that have a partially spiky and partially smooth appearance. Given our results in **Chapter 3**, the heterogeneous distribution of prM may be an important factor in controlling the fusogenic properties of a virus particle. It will be of interest to determine how many prM proteins per virus particle would still allow that virion to undergo membrane fusion.

ANTIBODY RESPONSE TO FLAVIVIRUS INFECTION

Analyses of patients serum samples and repertoire cloning of B-cells isolated from patients have provided insights into the polyclonal Ab response to flavivirus infections. These studies show that Abs raised in response to flavivirus infections are directed predominately against the E, prM and NS-1 proteins (Throsby *et al.*, 2006; Lai *et al.*, 2008; Dejnirattisai *et al.*, 2010). Interestingly, 90 % of the E-specific response represent weakly neutralizing Abs and therefore it is

generally assumed that they do not significantly contribute to protection (Throsby *et al.*, 2006; Oliphant *et al.*, 2006; Oliphant *et al.*, 2007; Lai *et al.*, 2008; Crill *et al.*, 2009; Dejnirattisai *et al.*, 2010). Indeed, these cross-reactive Abs have been postulated to be involved in enhancement of flavivirus infection (Pierson *et al.*, 2007; Cherrier *et al.*, 2009), as will be discussed later. Potently neutralizing MAbs against WNV and DENV have been previously mapped to a highly exposed epitope within DIII and have been considered to be crucial for *in vivo* protection.

Other studies have shown that anti-prM Abs can also provide passive protection against DENV infection in mice (Kaufman *et al.*, 1987; Falconar, 1999). It is unclear how Abs raised against prM might neutralize infection. Instead, they have recently been shown to enhance infection of immature DENV on FcR-bearing cells (Dejnirattisai *et al.*, 2010; Rodenhuis-Zybert *et al.*, 2010). Anti-NS1 Abs have been shown to protect by targeting NS-1 proteins expressed on the cell surface, thereby promoting the clearance of infected cells through FcR-dependent mechanisms (Chung *et al.*, 2006).

NEUTRALIZATION OF FLAVIVIRUS INFECTION: UNDERSTANDING THE ROLE AND FUNCTION OF ANTIBODIES

The modus operandi of an Ab critically depends on its binding epitope and considerable effort has been made to identify regions on the flavivirus virion that elicit potently neutralizing Ab responses. More particularly, many studies have focused on mapping of the binding epitopes of Abs that efficiently cross-neutralize all four DENV serotypes in attempts to guide dengue vaccine development. Mapping of the binding sites of potently neutralizing Abs has revealed that neutralizing epitopes can be found throughout the E-glycoprotein (Crill & Roehrig, 2001; Morita *et al.*, 2001; Goncalvez *et al.*, 2004; Nybakken *et al.*, 2005; Oliphant *et al.*, 2006; Oliphant *et al.*, 2007; Sukupolvi-Petty *et al.*, 2007; Lai *et al.*, 2007; Shrestha *et al.*, 2010; Sukupolvi-Petty *et al.*, 2010; Brien *et al.*, 2010) and may even span the E-homodimer interface (Throsby *et al.*, 2006; **Chapter 5**; Crill *et al.*, 2009; Sukupolvi-Petty *et al.*, 2010). In general, however, potently neutralizing Abs map to epitopes within DIII (Nybakken *et al.*, 2005; Oliphant *et al.*, 2006; Oliphant *et al.*, 2007; Sukupolvi-Petty *et al.*, 2007; Shrestha *et al.*, 2010; Brien *et al.*, 2010). X-ray crystallographic and yeast display epitope mapping studies have demonstrated that the potently neutralizing anti-WNV MAb E16 engages a highly accessible epitope on the lateral ridge of DIII (Nybakken *et al.*, 2005). A structurally analogous epitope on the lateral ridge of DIII was later identified on the DENV E-glycoprotein (Shrestha *et al.*, 2010; Brien *et al.*, 2010; Wahala *et al.*, 2010). Importantly, because of large sequence variation of residues within this epitope, distinct serotypes or genotypes are poorly neutralized by DIII lateral ridge MAbs. Intriguingly, compared to cross-reactive Abs, DIII Abs are present in much lower levels in human serum and several studies have now questioned their contribution to *in vivo* protection against WNV and DENV infections (Oliphant *et al.*, 2007; Wahala *et al.*, 2009). Recently, a number of studies have suggested the involvement of Abs binding to conformational epitopes such as the homodimer interface, in protection against flavivirus infection (Throsby *et al.*, 2006; **Chapter 5**; Wahala *et al.*, 2009; Sukupolvi-Petty *et al.*, 2010). Abs recognizing conformational epitopes have been shown to comprise approximately 30% of the Ab response to WNV infection (Throsby *et al.*, 2006), but are often overlooked in large IgG-screenings

because recombinant proteins instead of whole virions are used as antigen. In **Chapter 5**, we describe two highly neutralizing MAbs isolated from patients naturally infected with WNV, CR4354 and CR4348, that did not bind recombinant E in yeast-display mapping experiments, but readily bound whole virions. Importantly, binding was sensitive to exposure to low-pH, suggesting the involvement of a conformational epitope. Although CR4354 and CR4348 were shown to protect mice against lethal WNV challenge, the contribution of MAbs recognizing conformational epitopes to protection in humans remains to be determined. In contrast, more weakly neutralizing MAbs were found to be cross-reactive, recognizing more conserved epitopes predominantly on DI and DII (Oliphant *et al.*, 2006; Stiasny *et al.*, 2006; Lai *et al.*, 2008). The large differences in neutralization potency between type-specific and cross-reactive MAbs have been related to the surface accessibility of their respective epitopes (Sukupolvi-Petty *et al.*, 2007; Gromowski *et al.*, 2008).

Role of occupancy in neutralization

Neutralization of flaviviruses is a 'multi-hit' phenomenon and occurs when the number of Abs that bind the virion reaches a required threshold (Pierson *et al.*, 2007). The fraction of epitopes occupied by a MAb at a given concentration is a major determinant of the neutralizing potency of MAbs (Klasse & Sattentau, 2002). Many of the conserved epitopes recognized by cross-reactive MAbs are expected to be poorly exposed on the virion surface, limiting the number of sites available for engagement by MAbs. In addition, epitope availability may be limited further by the unconventional arrangement of E-dimer rafts on the virion. Within mature particles, the E-glycoprotein homodimers exist in three chemically distinct environments, resulting in differential surface accessibility of epitopes in each of these environments (Pierson *et al.*, 2007). Together, these factors critically alter the number of binding sites available for occupation by MAbs. It was found that neutralization of WNV by MAbs recognizing the highly accessible DIII lateral ridge requires engagement of less than half of the available epitopes (Pierson *et al.*, 2007). Conversely, many cross-reactive MAbs require occupancy levels exceeding 90% to reach the threshold for neutralization. The potency of MAbs that require high levels of occupancy for neutralization is strongly influenced by the maturation state of the virion (Nelson *et al.*, 2008). It has been postulated that the presence of uncleaved prM within partially immature particles alters the availability of epitopes that are predicted to be occluded within the mature virion. Indeed, virions with higher levels of prM were shown to be more sensitive to neutralization in non-FcR expressing cells by cross-reactive MAbs than mature virions (Nelson *et al.*, 2008).

Mechanisms of antibody neutralization: inhibition of membrane fusion

Abs are uniquely equipped to potently inhibit viral infection of target cells by directly binding to the virion. The textbook dogma states that Abs inhibit cellular entry by blocking interactions with the virus receptor. This view has proved somewhat outdated as Abs have been shown to be able to inhibit viral entry at many different stages including binding, internalization by endocytosis, membrane fusion, capsid uncoating and trafficking to the appropriate cellular location (reviewed by Klasse & Sattentau, 2002; Reading & Dimmock, 2007). In case of flaviviruses, MAbs have been described that specifically inhibit viral binding to non-Fc-receptor expres-

sing cells (Roehrig *et al.*, 1998; Hung *et al.*, 1999; Crill & Roehrig, 2001). Other MAbs were found to neutralize not only cellular attachment but also at a postattachment stage. Gollins and Porterfield (Gollins & Porterfield, 1986a) were the first to show that Abs are able to neutralize viral infection by inhibiting fusion of the viral envelope with the endosomal membrane. Ab-mediated neutralization of membrane fusion has since been shown to be a common mechanism amongst flaviviruses (Gollins & Porterfield, 1986a; Randolph & Stollar, 1990; Roehrig *et al.*, 1998; Butrapet *et al.*, 1998; Crill & Roehrig, 2001; Goncalvez *et al.*, 2004; Nybakken *et al.*, 2005; Stiasny *et al.*, 2007; Lai *et al.*, 2007; Chapters 4 and 5).

In **Chapter 4**, we show that the highly neutralizing anti-WNV MAb E16 potently inhibits membrane fusion with liposomes. Intriguingly, cryo-EM reconstructions have revealed that E16 inhibits an intermediate step in the fusion process, thereby locking the virion structure in a dead-end conformation upon low-pH exposure (Kaufmann *et al.*, 2009). The mechanism of inhibition proposed by Kaufmann and coworkers is in agreement with the observation that E16 neutralizes WNV at very low fractional occupancy (Pierson *et al.*, 2007). Indeed, the low stoichiometric threshold may result from this 'active' mode of inhibition; trapping a virion in a dead-end conformation may require initial binding of a single Ab whereas inhibition of cellular attachment likely requires engagement of all available receptor-binding sites (Lok *et al.*, 2008).

An important feature of fusion-inhibitory Abs is they may block viral infection irrespective of the mode of entry. Abs that exclusively neutralize infection through blockade of cellular attachment are not expected to block FcR-mediated entry of opsonized virus particles, possibly leading to enhancement of infection (as discussed in the next section). Regardless of the entry pathway, however, fusion with the endosomal membrane is an obligatory step in the flavivirus lifecycle and remains an important target for neutralization by fusion-inhibitory Abs.

ENHANCEMENT OF FLAVIVIRUS INFECTION: WHEN NEUTRALIZATION FAILS

Enhancement of infection may occur when the number of Abs engaging a virion is below the threshold required for neutralization. This threshold was shown to depend on epitope accessibility and Ab-affinity. Importantly, Abs that fail to meet the occupancy requirements for neutralization may still have bound in sufficient numbers to allow their opsonization into FcR-bearing cells. Ab-dependent enhancement of infection (ADE) was first described by Halstead and coworkers (Halstead & O'Rourke, 1977; Halstead *et al.*, 1977) and involves the enhanced uptake of opsonized DENV particles into monocytes, macrophages and dendritic cells. The higher viral loads resulting from increased numbers of infected cells have been postulated to be crucial in the development of severe disease.

Influence of particle maturation status

It has become clear that the maturation status of the flavivirus particle greatly influences the availability of epitopes on the E-glycoprotein (Nelson *et al.*, 2008; Cherrier *et al.*, 2009) Regions on DI and DII that are occluded within the mature virion are expected to be more accessible within the context of immature virions. In fact, recent cryoEM analyses of the cross-reactive

MAb E53, targeting the fusion loop, in complex with wild-type or immature virions indicate that Fab-fragments of E53 fail to engage mature particles whereas they readily bind (partially) immature particles (Cherrier *et al.*, 2009). As this MAb does not readily bind mature particles, it is unlikely to reach the occupancy requirements for neutralization. This has indeed been shown for E60, another cross-reactive MAb directed against the fusion loop. Several studies have demonstrated that E60 fails to fully neutralize infection, even at saturating concentrations (Oliphant *et al.*, 2006; Pierson *et al.*, 2007; Nelson *et al.*, 2008). Interestingly, the presence of a virus fraction resistant to neutralization by E60 was also observed in fusion measurements with liposomes (**Chapter 4**). At the highest concentration tested, under conditions of MAb excess, approximately 30% of the virions within the population still underwent fusion. The apparent heterogeneity within the population likely relates to the maturation state of individual virions, although this warrants further investigation. If we assume that the fully mature virions within the population are not susceptible for neutralization by E60, and thus represent the resistant fraction of 30%, it would follow that the neutralized fraction would consist of particles more susceptible for neutralization: partially immature particles. The observations therefore suggest that partially immature particles are capable of fusion with liposomal membranes. The notion that partially immature particles are infectious is supported by Nelson and coworkers (Nelson *et al.*, 2008). In their study, they conclude that particles containing higher levels of prM are more readily neutralized by cross-reactive MAbs, an observation implicitly implying that these particles are themselves infectious. Also, anti-prM MAbs have been shown to be neutralizing, albeit to a low extent, suggesting that binding of these MAbs to the prM portion of partially immature particles blocks their infectivity through mechanisms that remain to be defined (Kaufman *et al.*, 1989; Vazquez *et al.*, 2002; Dejnirattisai *et al.*, 2010). The ability of cross-reactive Abs, which are highly abundant within polyclonal serum, to neutralize flavivirus infection is therefore highly dependent on the prM content of individual particles.

Influence of antibody affinity

Detailed epitope mapping of numerous MAbs raised in mice against WNV and DENV has revealed that Ab-binding epitopes consist of only a limited number of residues. Anti-WNV MAb E16 was found to engage a conformational epitope formed by four discontinuous strands on the lateral ridge of DIII (Nybakken *et al.*, 2005). Yeast surface display epitope mapping identified four central residues that are critically involved in binding of E16. An analogous DENV MAb 3H5, also associated with strong neutralization, was mapped to a structurally identical region on the DIII lateral ridge of DENV-2 (Sukupolvi-Petty *et al.*, 2007). Binding of this MAb was shown to rely on three central residues at two adjacent strands. The notion that binding of potently neutralizing MAbs depends on only a small number of residues has considerable consequences for our understanding of Ab neutralization. One of the main implications is that small changes within the epitope can result in large differences Ab-affinity, which critically influences binding specificity and neutralization capacity. This was recently exemplified by several studies investigating the ability of DIII lateral ridge MAbs to potently neutralize multiple DENV-1, DENV-2 and DENV-3 genotypes (Shrestha *et al.*, 2010; Sukupolvi-Petty *et al.*, 2010; Brien *et al.*, 2010; Wahala *et al.*, 2010). Despite the high degree of sequence conserva-

tion between these genotypes, large differences in neutralization potency and *in vivo* protection were observed. These differences were caused by naturally occurring sequence variations within the epitope. In addition, Shrestha and coworkers postulated that genotype-specific variation in residues outside the binding epitope may contribute to altered neutralization potency (Shrestha *et al.*, 2010). Indeed, changes in residues located outside an epitope have been shown to affect neutralization without abrogating binding of the MAb to its epitope (Goncalvez *et al.*, 2004; **Chapter 5**). To conclude, affinity of binding is an important factor because it critically influences MAb engagement of virions. In heterologous DENV infections, MAbs are likely to bind poorly and cause enhancement of infection. Alternatively, low-affinity MAbs may be internalized along with the particle and might dissociate upon exposure to low-pH, allowing the virion to initiate infection.

Antibody-mediated uptake of immature flaviviruses

The relevance of prM-containing flavivirus particles has been demonstrated by several recent papers. Importantly, Rodenhuis-Zybert and coworkers found that fully immature DENV particles opsonized with anti-prM MAbs are capable of infectious entry into FcR-bearing cells (Rodenhuis-Zybert *et al.*, 2010). They observed that furin-cleavage upon endocytosis of opsonized particles was crucial for infectivity. This notion was extended to WNV in **Chapter 6**, where high infectious titers were recovered upon infection of murine macrophages with anti-prM opsonized immature WNV particles. Furthermore, infectious virus could be recovered from blood serum and brain of mice infected with fully immature WNV complexed with anti-prM MAb, while no virus was found when the mice received immature virus alone (**Chapter 6**).

Not only prM Abs but also E Abs are able to interact with prM-containing flavivirus particles. In **Chapter 7**, the entry pathway of fully immature DENV and WNV complexed with the cross-reactive anti-E MAb E53 was investigated. An important finding of this study is that E53 indeed supported the infectious entry of fully immature DENV and WNV. However, MAb E53 failed to promote the infectious properties of immature DENV on human leukemia K562 cells while it readily enhanced infectivity on murine P388D1 macrophages over a broad range of concentrations. It is demonstrated that opsonization of immature DENV with E53 shifts the optimum pH at which furin-mediated cleavage of prM (a required step in the entry of fully immature particles) takes place to lower pH values, likely explaining the differential enhancement in the two cell lines. It is not well understood what distinguishes DENV from WNV in this respect, although it is interesting to note that the E53 epitope on both viruses differs by one residue. This difference may cause considerable variation in binding affinity, either at neutral or low pH, allowing the MAb to dissociate from WNV but not DENV. The Ab-mediated blockade of furin cleavage of immature particles is a novel level of neutralization, relevant exclusively to the infectious entry of prM-containing virions and should be investigated in more detail.

Another mechanistical aspect of the entry of immature particles that remains to be clarified is how the pr-peptide dissociates upon furin-cleavage of the prM protein, rendering the particle fusogenic. According to current models of the flavivirus maturation process, the pr-peptide is released from the virion upon exposure to neutral pH (Yu *et al.*, 2008). Paradoxically, the immature particle encounters increasingly lower pH values upon endosomal uptake and we

propose that exposure to acidic pH may trigger the release of the pr-peptide. Functional studies suggest that pr-dissociation does not occur at pH levels as low as 5.5 (Yu *et al.*, 2009). However, it is well known that phagosomes are acidified upon internalization of cargo and subsequently fuse to lysosomes (pH 5.0), making it likely that opsonized complexes are exposed to pH values below 5.5 (as reviewed by: Mellman *et al.*, 1986). Indeed, recent preliminary observations of entry of opsonized immature DENV particles into living cells suggest that the time-to-fusion of these complexes is somewhat prolonged compared to non-opsonized wild type DENV (unpublished observations Ayala-Nunez, Wilschut, Smit). This observation would be in line with current models of acidification of endocytic vesicles where the initial acidification to pH 6 occurs within 5 minutes upon internalization, followed by a subsequent slow acidification to pH 5 within 30 - 40 minutes (Mellman *et al.*, 1986). Analyses of the entry kinetics of opsonized immature particles, perhaps coupled with intra-endosomal pH measurements, are therefore expected to provide important insights into the functional mechanism of maturation upon entry.

Recent studies have implicated the involvement of prM-containing particles in the enhanced disease associated with secondary DENV infections through association with non-neutralizing MAbs (Dejnirattisai *et al.*, 2010; Rodenhuis-Zybert *et al.*, 2010; Schmidt, 2010). It is tempting to speculate that activation of the infectious properties of these otherwise non-infectious particles contributes to high viremia in secondary infections by increasing the number of infected cells. The identification of the functional aspects described above in this novel entry mechanism are of significant importance and may lead to new strategies to prevent the infectious uptake of partially immature particles.

PERSPECTIVES

Considerations for vaccine design

An important goal of flavivirus research has been to guide the development of safe and efficient vaccines against DENV. Recent advances in our understanding of the mechanisms of flavivirus neutralization and how failure to neutralize relates to enhancement of infection have revealed some important aspects that have not yet been considered in vaccine strategies. One of the major findings is the possible involvement of prM-containing DENV particles in disease development through FcR-dependent mechanisms. Abs directed against the prM protein were found to be highly cross-reactive and weakly neutralizing. It has therefore been suggested that DENV antigens used in the vaccine formulation should be adapted such that they do not readily give rise to these weakly neutralizing MAbs (Dejnirattisai *et al.*, 2010). A vaccine consisting of merely fully mature virions may therefore meet these conditions.

Antibodies as antiviral therapeutics

The identification of potently neutralizing MAbs that provide robust protection against WNV in rodent challenge models has prompted researchers to explore their possible use as therapeutics in humans. As immunocompromised individuals are more likely to develop WNV encephalitis, a therapeutic should potently control viral spread and allow rapid clearance of viruses from the central nervous system. MAb E16 has been demonstrated to protect mice from lethal WNV challenge when administered as a single dose at 5 days postinfection. The application of

humanized E16 as a therapeutic against WNV is currently being investigated in clinical trails (Diamond, 2009). Interestingly, several MAbs were recently described to potently inhibit infection of influenza and HIV through blockade of membrane fusion (Ekiert *et al.*, 2009; Alam *et al.*, 2009). These MAbs were shown to bind a transient structural intermediate that is only exposed upon fusion. Importantly, the epitopes engaged by these MAbs are highly conserved because of their critical role in the fusion process. As a result, these MAbs are broadly cross-reactive, recognizing a wide range of strains and variants. It would be of interest to identify analogous fusion-inhibitory anti-flavivirus MAbs as they may represent a novel class of highly potent antiviral therapeutics with the ability to cross-neutralize multiple serotypes and genotypes.

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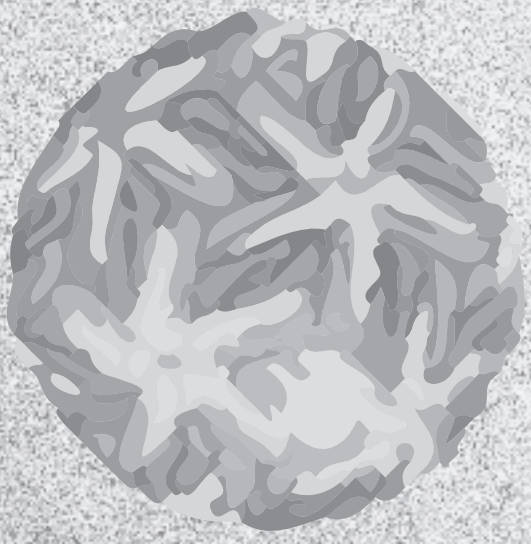
Nederlandse samenvatting



Inleiding

Het dengue virus (DENV) en het West Nijl virus (WNV) behoren tot de *Flaviviridae*, een familie van kleine sferische virussen met een positief-strengs RNA genoom. Mensen kunnen worden besmet met het virus wanneer ze worden gestoken door een muskiet die drager is van het virus. De dengue muskiet komt voor in bijna alle subtropische gebieden van de wereld waardoor naar schatting de helft van de wereldbevolking risico loopt besmet te raken met het dengue virus. Een beet van een besmette muskiet leidt in de meeste gevallen niet tot merkbare ziekteverschijnselen al kunnen ook milde tot ernstige ziektebeelden ontstaan. Het WNV veroorzaakte in recente uitbraken in de Verenigde Staten in ongeveer 1 op de 150 gevallen virale encephalitis (ontsteking van de hersenen). De patiënt loopt hierbij het risico op chronische neurologische aandoeningen en komt in zo'n 10% van de gevallen te overlijden. Het ziektebeeld van het DENV is meer complex. In de meeste gevallen leidt een besmetting tot dengue koorts, ook wel knokkelkoorts genoemd, welke na 4 – 7 dagen afzwakt. In sommige gevallen echter, treden na de koortsperiode ernstige bloedingen (lekkende vaten) op waardoor de patiënt in een shocktoestand terecht kan komen, met soms fatale afloop. Jaarlijks worden naar schatting 50 tot 100 miljoen mensen geïnfecteerd met het dengue virus, waarvan 500.000 tot 1.000.000 mensen de ernstige vorm van de ziekte ontwikkelen en deze kent in 20.000 gevallen een fataal beloop. Er zijn tot op heden geen vaccins of geneesmiddelen tegen het DENV of WNV, de behandeling van patiënten is vooral ondersteunend. Op dit moment is de enige effectieve manier om het aantal besmettingen te beperken het bestrijden van de muskiet.

Doel van dit proefschrift

In recente jaren is duidelijk geworden dat antistoffen een belangrijke rol spelen in de natuurlijke afweer tegen flavivirusinfecties. Voor de meeste flavivirussen geldt dat eerdere blootstelling aan het virus leidt tot levenslange bescherming tegen herinfectie. Deze bescherming is toe te schrijven aan de aanwezigheid van antistoffen. Paradoxaal genoeg kan de aanwezigheid van antistoffen in het geval van het DENV juist leiden tot een verergerd ziektebeeld. Antistoffen hebben vele verschillende functies binnen het afweersysteem. Onze studies richten zich op de interactie van het virus met een gastheercel, en hoe dit proces door antistoffen beïnvloedt wordt. Het belangrijkste doel van de studies beschreven in dit proefschrift is dan ook om een beter inzicht te verkrijgen in enerzijds het proces waarop het virus een gastheercel infecteert en anderzijds het mechanisme waarop antistoffen dit proces kunnen beïnvloeden.

Studies beschreven in dit proefschrift

Het virus komt na een beet van een besmette muskiet het lichaam binnen en zal zich uiteindelijk hechten aan een gastheercel. Het virusdeeltje wordt vervolgens opgenomen in een transportblaasje, waarin het zich laat meevoeren naar het binnenste van de cel. Het milieu binnenin het transportblaasje wordt geleidelijk steeds zuurder, en uiteindelijk zou het virusdeeltje onder invloed hiervan worden afgebroken. De zure omgeving is echter een stimulus voor het virus om uit het blaasje te ontsnappen via een zogenaamd fusieproces. Tijdens het fusieproces versmelten de omhulsels van het virus en het zure blaasje met elkaar, waarbij het genetisch materiaal van het virus vrijkomt binnenin de cel. Na het vermenigvuldigen van het RNA worden nieuwe zogenoemde immature virusdeeltjes gevormd. Deze virusdeeltjes ondergaan een maturatieproces

waarbij het pre-membraan (prM) eiwit wordt geknipt in een membraan (M) eiwit, dat gebonden blijft aan het virus en een kleiner pr-peptide, dat vrijkomt nadat het virus de cel verlaat. Mature flavivirusdeeltjes bevatten daarom enkel het M en E eiwit in de eiwitmantel. Het is echter bekend dat zo'n 30% van het virus dat vrijkomt uit cellen geïnfecteerd met het DENV of WNV, de immature vorm heeft.

In **hoofdstuk 3** is het fusieproces van WNV nader onderzocht. Dit hebben we gedaan door het fusieproces in het laboratorium te simuleren. Hierbij werd gebruik gemaakt van zogenaamde liposomen. Dit zijn sferische membraantjes bestaande uit verschillende fosfolipiden, aangevuld met sfingolipiden en cholesterol, waarvan de samenstelling te vergelijken is met het omhulsel van een transportblaasje. De virusdeeltjes werden 'geladen' met een fluorescente merker en vervolgens in een reactievaatje gemengd met liposomen. Bij aanzuring van het reactievaatje versmelten het virusomhulsel en het liposoom, waarbij de fluorescente merker zich vanuit het virusomhulsel verspreidt over het liposoom. De afname van de fluorescentie-intensiteit die gepaard gaat met de herverdeling van de merker over het liposoom kan rechtstreeks worden gevolgd met een fluorometer, en geeft zowel kinetische als kwantitatieve informatie over de fusie-activiteit van het virus. Op deze wijze werd vastgesteld dat de versmelting van het WNV met liposomen een erg snel proces is dat zich binnen 3 seconden voltrekt. Ook werd duidelijk dat versmelting reeds onder mildzure condities (pH <6.7) plaatsvond. Verder bleek de aanwezigheid van cholesterol in het liposoom het fusieproces te faciliteren, maar was er geen absolute vereiste voor cholesterol of sfingolipiden. Daarnaast werd aangetoond dat immatuur WNV niet in staat is te fuseren met liposomen. Door vervolgens deze immature deeltjes te behandelen met een enzym dat het prM eiwit op de eiwitmantel knipt (en zo in feite het maturatieproces in een reageerbuis te simuleren), konden deze virusdeeltjes weer fusie-actief worden gemaakt. Deze conclusies stellen ons in staat het fusieproces van het WNV te vergelijken met dat van andere virussen, en geven meer inzicht in het opnameproces van flavivirussen.

Hoofdstuk 4 beschrijft de moleculaire basis voor de bescherming die de antistof 'E16' biedt tegen het WNV. Van dit antistof is in muizen aangetoond dat toediening ervan bescherming geeft tegen een anderszins dodelijke WNV infectie. Om beter te begrijpen waarom dit antistof zo effectief is, hebben we gekeken naar de interactie van het WNV met de gastheercel in aanwezigheid van E16. Met behulp van microscopie is aangetoond dat E16 niet, zoals veel andere antistoffen, voorkomt dat het virus aan het celoppervlak bindt. Virusdeeltjes met daarop E16 antistoffen werden aangetroffen in de transportblaasjes binnenin de cel. In aanwezigheid van de antistof bleken de virusdeeltjes echter niet uit de transportblaasjes te kunnen ontsnappen. De hypothese dat E16 het fusieproces remt werd verder onderzocht door de fusieactiviteit van het WNV met liposomen in aanwezigheid van E16 te meten. Hieruit bleek dat E16 een sterk remmende werking had op de fusie-activiteit van WNV en dat bij hoge concentraties het fusieproces zelfs compleet geblokkeerd was. Deze experimenten wijzen erop dat de antistof E16 WNV infectie blokkeert door te voorkomen dat het virus uit de transportblaasjes kan ontsnappen. Op dit moment wordt de effectiviteit van therapeutische toediening van antistof E16 bij mensen na WNV infectie onderzocht in klinische studies.

Hoofdstuk 5 beschrijft de karakterisatie van antistoffen 'CR4354' en 'CR4348' welke zijn geïsoleerd vanuit het bloed van patiënten die een infectie met het WNV hebben opgelopen. Deze antistoffen bleken zeer effectief in het beschermen van muizen tegen een dodelijke WNV

infectie. Door gebruik te maken van het liposomale modelsysteem kon worden vastgesteld dat beide antistoffen net als E16 in staat waren het fusieproces van WNV te remmen. Om te begrijpen op welke wijze deze remming plaatsvond, werd bepaald waar de antistoffen precies binden op het virusoppervlak. Omdat de mantel van het flavivirusdeeltje voornamelijk bestaat uit envelop (E) eiwitten, zijn de meeste antistoffen tegen dit eiwit gericht. CR4354 en CR4348 herkenden echter niet een bepaalde regio binnen dit E-eiwit (zoals het merendeel van de reeds gekarakteriseerde antistoffen), maar bonden E-eiwitten alleen als deze zich in een bepaalde positie tot elkaar bevonden. Omdat het fusieproces afhankelijk is van de samenwerking van meerdere E-eiwitten op het virusdeeltje, kan worden veronderstelt dat binding van de hier beschreven antistoffen op deze posities binnen het virusdeeltje dit proces blokkeert.

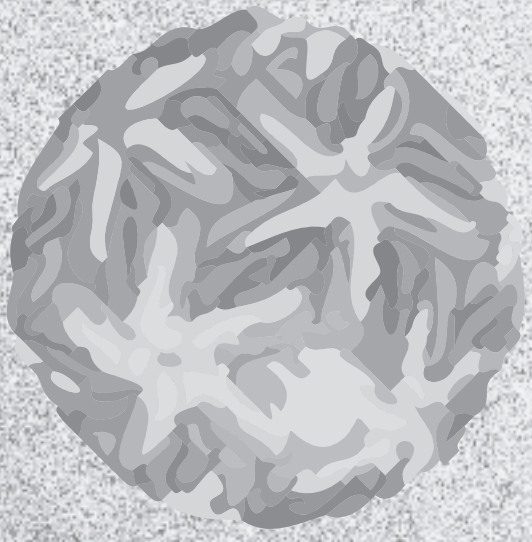
Voor het DENV is onlangs aangetoond dat immature virusdeeltjes in aanwezigheid van antistoffen tegen het prM-eiwit op bepaalde celtypes bijna net zo infectieus zijn als mature deeltjes. Het virusdeeltje met daaraan antistoffen gebonden wordt op deze celtypes namelijk opgenomen via een speciale opnameroute waarbij de antistoffen binden aan zogenaamde Fc-receptoren, receptoren die antistoffen herkennen. De bedoeling hiervan is dat hetgeen gebonden is aan de antistof binnenin de cel wordt afgebroken. Het virus weet hier echter aan te ontsnappen door binnenin het transportblaasje alsnog het maturatieproces te ondergaan, waarna het fusie-actief wordt en uit het blaasje kan ontsnappen. Dit verschijnsel wordt ook wel antistof-gemedieerde opname genoemd, en vindt alleen plaats op celtypes van het immuunsysteem.

In **hoofdstuk 6** wordt aangetoond dat immature West Nijl virusdeeltjes, waarvan bekend is dat ze niet infectieus zijn in afwezigheid van antistoffen (**hoofdstuk 3**), in muizen een dodelijke infectie kunnen veroorzaken in combinatie met antistoffen gericht tegen het prM-eiwit. Deze studie toont aan dat immature deeltjes, in aanwezigheid van antistoffen, een rol kunnen spelen in het ziekteproces van WNV.

Hoofdstuk 7 laat zien dat ook antistoffen gericht tegen het E-eiwit kunnen leiden tot antistof-gemedieerde opname van immature flavivirusdeeltjes. Door gebruik te maken van cellen met Fc-receptoren, die complexen van antistoffen en virussen kunnen opnemen, werd onderzocht hoe de antistof 'E53' het opnameproces van immatuur DENV en WNV kan faciliteren. Hieruit bleek dat het immature WNV in alle onderzochte celtypes weer infectieus werd, terwijl immature dengue virusdeeltjes niet in alle celtypes infectieus werden. Door het maturatieproces in het lab te simuleren bleek dat het prM-eiwit van het immature DENV minder efficiënt werd geknipt dan immatuur WNV, onder de condities zoals die aanwezig zijn binnen transportblaasjes in de cel. Deze studie maakt duidelijk dat kleine verschillen in de structuur van twee virussen belangrijke consequenties kunnen hebben op het maturatieproces bij antistof-gemedieerde opname van immature flavivirusdeeltjes.

Hoofdstuk 8 geeft een samenvatting van de studies beschreven in dit proefschrift. Met deze studies is een bijdrage geleverd aan het inzichtelijk maken van de interacties tussen antistoffen en flavivirussen, en hoe deze de infectie van een gastheercel beïnvloeden. Deze kennis is van belang voor de gerichte ontwikkeling van antivirale geneesmiddelen en vaccins tegen flavivirussen met als uiteindelijk doel het voorkomen van het ziekteleed dat door deze virussen wordt veroorzaakt.

Dankwoord



DANKWOORD

Eindelijk is daar dan de eindstreep in zicht! Met dit laatste hoofdstuk is het boekje een feit, en dat voelt toch wel heel goed. Ik ben dank verschuldigd aan veel verschillende mensen die allen hebben bijgedragen aan de totstandkoming van dit proefschrift.

Allereerst wil ik mijn promotor Jan Wilschut en co-promotor Jolanda Smit bedanken.

Jan, ik heb je begeleiding tijdens de vele reguliere en net zo vele ingelaste besprekingen enorm gewaardeerd.

Jolanda, jij was de drijvende kracht gedurende mijn promotietraject en hiervoor wil ik je bijzonder bedanken. Op belangrijke momenten was jouw aanstekelijke optimisme altijd de impuls om weer met frisse moed verder te gaan. Bedankt voor de manier waarop je me altijd hebt geholpen mijn experimenten te doorgronden, ik heb veel geleerd van je inzicht en expertise.

De leescommissie, bestaande uit Prof. Boddeke, Prof. van Oijen en Prof. Wiertz ben ik zeer erkentelijk voor het beoordelen van dit proefschrift.

Het was fantastisch om mijn promotieonderzoek te kunnen doen bij de Moleculaire Virologie. Ik wil al mijn collega's bedanken voor de goede werksfeer en wens iedereen het allerbeste toe in de toekomst.

Ik heb het enorm leuk gevonden om deel uit te maken van de Dengue groep, in het begin een klein gezelschap met Hilde, Iza, Heidi en Jolanda, die later uitgroeide tot een grotere groep toen Júlia, George en Vanesa erbij kwamen. De dinsdagochtendbesprekingen: vaak verhitte discussies in de vroege ochtend, het gezamenlijk inrichten van het ML-III lab, de gezellige sfeer tijdens het werken op het ML-III, maar ook de gedrevenheid binnen de groep, allemaal herinneringen waar ik met veel plezier aan terugdenk.

Hilde, Iza, Heidi, Júlia, George en Vanesa: bedankt!

Mijn kamergenoten Iza, Mateusz, Shafique en George, I would like to thank all of you for the wonderful atmosphere in our room. It has always been a great place to work hard, and a perhaps better place to take a well deserved break. Mateusz, wij komen jou, Kasia en Zosia snel eens opzoeken in Maastricht.

I would like to express my gratitude to our collaborators at the Washington University School of Medicine, St. Louis, USA, Crucell Holland, The Netherlands, and the Yale University School of Medicine, New Haven, USA.

Natuurlijk is er ook een leven na het promotieonderzoek. Ik voel me enorm thuis op mijn werkplek bij de Moleculaire Genetica en ik wil al mijn collega's daarvoor bedanken!

First and foremost, this book is dedicated to my family. Pap, mam, Jacintha, opa & oma, bedankt!

Carina, my love, I could not have done this without your smiles, love, patience and support. Thank you for everything!! As I close this chapter in our lives, I'm looking forward to many more chapters to come together with you and Hugo. *I love you.*

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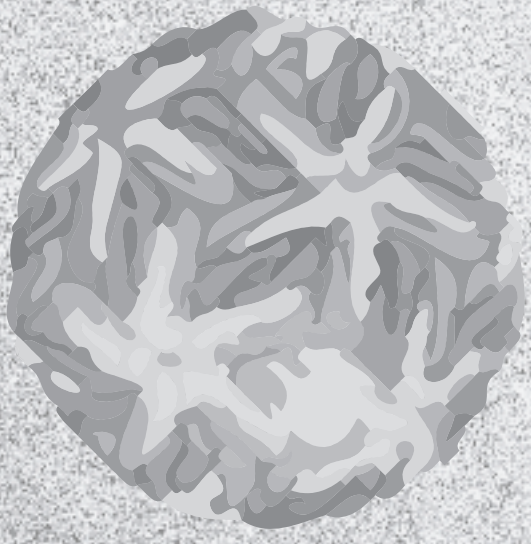

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